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VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

FACULTY OF CHEMISTRY
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INSTITUTE OF MATERIALS SCIENCE
ÚSTAV CHEMIE MATERIÁLŮ

MODIFICATION OF THERMOSENSITIVE COPOLYMER WITH BIOACTIVE SUBSTANCES FOR MEDICAL APPLICATIONS

MODIFIKACE TERMOCITLIVÉHO KOPOLYMERU BIOAKTIVNÍMI LÁTKAMI PRO MEDICÍNSKÉ APLIKACE

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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1. Literární rešerše na téma termocitlivé kopolymery a jejich modifikace
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ABSTRACT

Biodegradable synthetic polymers bring many advantages over other materials for the utilization in the field of regenerative medicine and tissue engineering. The most important advances involve the capability of optimalizing mechanical or chemical properties and the degradation kinetics. Especially polyesters are interesting because of their simple biodegradation. They undergo the hydrolysis of ester linkage and the degradation products are metabolized without harmful effects.

Diploma thesis is focused on synthetic biodegradable PLGA-PEG-PLGA triblock copolymers based on poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(ethylene glycol) (PEG) that belong to the group of biodegradable polyesters. The content of hydrophilic and hydrophobic parts of polymer chain induces the amphiphilic character. Prepared triblock copolymers are capable of forming hydrogel by physical cross-linking in consequence of their amphiphilic character. These materials have noticed significant interest in the field of medical sciences.

Theoretical part describes hydrogels, physical cross-linking of amphiphilic block copolymers and the mechanisms of degradation. Description of PLGA-PEG-PLGA triblock copolymers is divided on PLGA copolymers, PEG and their physico-chemical properties. Reliable knowledge of chemical functionalization by succinic anhydride, folic acid and itaconic anhydride is presented. Dopamine is introduces as a linker and the most important bioactive substances are mentioned.

Experimental part presents certain methods of synthesis that lead to functionalization and modification of PLGA-PEG-PLGA triblock copolymers. Functionalization by itaconic anhydride was proceeded to obtain functionalized copolymer with both ends capped by reactive double bonds and carboxylic groups. The double bonds enable to form chemical cross-links and the end-capped carboxylic groups offer the opportunity to modify it by biologically active compounds. The modification by bioactive substances L-lysine and butylamine enriches the polymer network and linker dopamine provides the versatility of attached bioactive substances, their stabilization and the maintenance of its biological activity. Final products were characterized by the means of ^{1}H NMR, FTIR and DRA analysis.

Functionalization was carried out in a bulk with higher amount of bonded itaconic acid 79.4 mol % and subsequent modifications were proceeded in aqueous solution, organic solution or in a bulk. The most effective method of modification was synthesis in organic solution with solvent N,N-dimethylformamide with activating system dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine. The highest amount of bonded dopamine was 18.6 mol %, the highest amount of attached butylamine was 7.8 mol % and L-lysine was not bonded at all.

KEYWORDS:

Thermosensitive biodegradable copolymer, chemical functionalization, itaconic anhydride, modification, dopamine, bioactive substance, butylamine, L-lysine, synthesis, physical cross-linking.

ABSTRAKT

Biodegradabilné syntetické polyméry nesú vlastnosti, ktoré ich zvýhodňujú oproti iným materiálom používaným na poli regeneratívnej medicíny a tkanivového inžinierstva. Najdôležitejšie výhody zahŕňajú schopnosť prispôsobovať mechanické a chemické vlastnosti aj kinetiku degradácie. Obzvlášť polyestery sú zaujímavé z pohľadu na ich biodegradáciu. Podliehajú hydrolýze, počas ktorej dochádza k štiepeniu esterových väzieb a degradačné produkty sú metabolizované bez akýchkoľvek škodlivých účinkov.

Diplomová práca je zameraná na syntetické biodegradabilné triblokové kopolyméry PLGA-PEG-PLGA s obsahom kyseliny polymliečnej (PLA), kyseliny polyglykolovej (PGA) a polyetylénglykolu (PEG), ktoré patria do skupiny biodegradabilných polyesterov. Obsah hydrofilnej a hydrofóbnej zložky polymérneho reťazca spôsobuje amfifilný charakter kopolyméru. Pripravené triblokové kopolyméry sú schopné tvoriť hydrogél pomocou fyzikálneho sieťovania v dôsledku ich amfifilného charakteru. Tieto materiály zaznamenali významný záujem vo vedeckej oblasti.

Teoretická časť diplomovej práce všeobecne popisuje hydrogély, bližšie sa venuje fyzikálnemu sieťovaniu amfifilných blokových kopolymérov a mechanizmom degradácie. Podrobný popis triblokového kopolyméru PLGA-PEG-PLGA je rozdelený na PLGA kopolyméry, PEG a ich fyzikálno-chemické vlastnosti. Zahrnuté sú aj poznatky o chemickej funkcionalizácii anhydridom kyseliny jantárovej, anhydridom kyseliny itakonovej a kyselinou listovou. Dopamín je prezentovaný ako spájací faktor a spomenuté sú taktiež najdôležitejšie bioaktívne látky.

Experimentálna časť sa zaoberá konkrétnymi metódami syntézy, ktoré viedli k funkcionalizácii a modifikácii triblokových kopolymérov PLGA-PEG-PLGA. Funkcionalizáciou anhydridom kyseliny itakonovej bol získaný kopolymér s oboma koncami obohatenými o reaktívne dvojité väzby a karboxylové funkčné skupiny. Dvojité väzby umožňujú chemické sieťovanie a koncové karboxylové skupiny ponúkajú možnosť modifikácie kopolyméru biologicky aktívnymi látkami. Modifikácia bioaktívnymi látkami L-lyzínom a butylamínom obohacuje polymérnu sieť a dopamín v roli spojovacieho faktoru poskytuje univerzálnosť v naväzovaní bioaktívnych látok, stabilizuje ich a zabezpečuje zachovanie biologickej aktivity naviazaných bioaktívnych látok predĺžením reťazca. Výsledné produkty boli charakterizované pomocou ^{1}H NMR, FTIR a DRA analýz.

Funkcionalizácia anhydridom kyseliny itakonovej bola prevádzaná v tavenine. Podarilo sa dosiahnuť vyššieho množstva naviazanej kyseliny itakovovej s hodnotou 79,4 mol % a následné modifikácie boli prevádzané vo vodnom roztoku, organickom roztoku a taktiež v tavenine. Bolo zistené, že najefektívnejšia metóda modifikácie bola syntéza v organickom roztoku s rozpúšťadlom N,N-dimetylformamidom a aktivačným systémom dicyklohexylkarbodiimid/4-(dimetylamino)pyridínom. Najvyššie množstvo naviazaného dopamínu bolo 18,6 mol %, najvyššie množstvo naviazaného butylamínu bolo 7,8 mol % a L-lyzín sa naviazať nepodarilo.

KĽÚČOVÉ SLOVÁ:

Termocitlivý biodegradabilný kopolymer, chemická funkcionalizácia, anhydrid kyseliny itakonovej, modifikácia, dopamín, bioaktívna látka, butylamín, L-lyzín, syntéza, fyzikálne sieťovanie.

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DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete.

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Student's signature

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1 INTRODUCTION

Human body is complicated system in which is difficult to treat even replace some parts in a safe, appropriate and acceptable manner. Used materials have to dispose of several important properties such as biodegradability and biocompatibility. Biocompatible material is able to be in contact with living tissue in the body without influencing it by undesirable or harmfull effects [1]. Biodegradable material undergoes the degradation at physiological conditions and produces biocompatible and toxicologically safe by-products eliminated via common metabolic pathways [2]. These materials represent the group named biomaterials and synthetic biodegradable polymers comprise large part of the biomaterial group.

Synthetic biodegradable polymers have a great applicable potential in the field of tissue engineering. They offer many advantages over other materials such as the ability to set up mechanical properties or the degradation rate. Attractive feature is the capability to fabricate them into different shapes according to special needs of applications. Moreover, they can be chemically modified by functional groups to enlarge the field of application.

Synthetic copolymers based on poly(lactic acid), poly(glycolic acid) and poly(ethylene glycol) belong to the group of biodegradable polyesters. They degrade easily via hydrolysis of ester links, degradation products are metabolised and resorbed. The content of hydrophilic and hydrophobic parts of polymer chain induces the ability to form thermosensitive hydrogels. At low temperatures, copolymers are dissolved in water and at higher temperatures can form a viscous gel. This fact is widely utilized in drug delivery systems [3].

Diploma thesis is focused on poly[(D,L-lactic acid)-co-(glycolic acid)]-b-poly(ethylene glycol)-b-poly[(D,L-lactic acid)-co-(glycolic acid)] (PLGA-PEG-PLGA) copolymer functionalized by itaconic acid (ITA). The ITA functionalization brings two end-capped carboxylic groups, two reactive double bonds and the ability to form physical and chemical cross-links. The modification by bioactive substances L-lysine (Lys) and butylamine (ButA) enriches the polymer network and linker dopamine (DOPA) provides the versatility of attached bioactive substances, their stabilization and the maintenance of its biological activity. Final products can led to the improvement of the drug delivery systems in the field of tissue engineering.

2 THEORETICAL PART

The ambition to enhance the life standard forces contemporary science to develop new materials that meet a number of demanding requirements. The improvement has resulted in polymers with unique properties. Knowledge from all research fields has made polymers smarter and more effective [4].

2.1 Hydrogels

In the centre of scientific interest are hydrogels. Hydrogel can be modified to various properties that make it suitable for utilization in natural, applied or medical sciences. Hydrogels play the prime role of the importance in biomedical and health care. The improvement of this material is very attractive and promising topic among scientists and researchers nowadays.

Hydrogel is defined as a polymeric network with three-dimensional structure and hydrophilic character capable of absorbing large volume of water or biological fluids. Described material swells but does not dissolve in the presence of water. In general, the bottom limit of hydrogel´s absorbion is 20 % and the amount of absorbed water depends on the hydrophilic character and density of polymeric network. Hydrophilic groups such as hydroxyl (-OH), carboxamide (-CONH-, -$CONH_2$-) and sulfo groups (-SO_3H) are responsible for high affinity to water, the change of chemical structure and thus forming the hydrogel.

Classification of hydrogels is based on different parameters, features and characteristics. In accordance to source we can distinguish natural and synthetic origin of hydrogels. Recently, synthetic hydrogels are applied mostly instead of natural ones because of their long service life, high capacity of water content and high level of gel strength. Synthetic polymers in hydrogels have well-defined structures and properties that can be modified in many different ways to obtain tailor-made characteristics.

The metod of preparation divides hydrogels into homopolymeric or copolymeric hydrogels, physical structure to amorphous, semicrystalline or crystalline and character of side groups may be neutral or ionic. In according to type of cross-linking, the polymeric network is formed by physical, chemical or biological cross-linking of single polymer chains (Fig. 1)

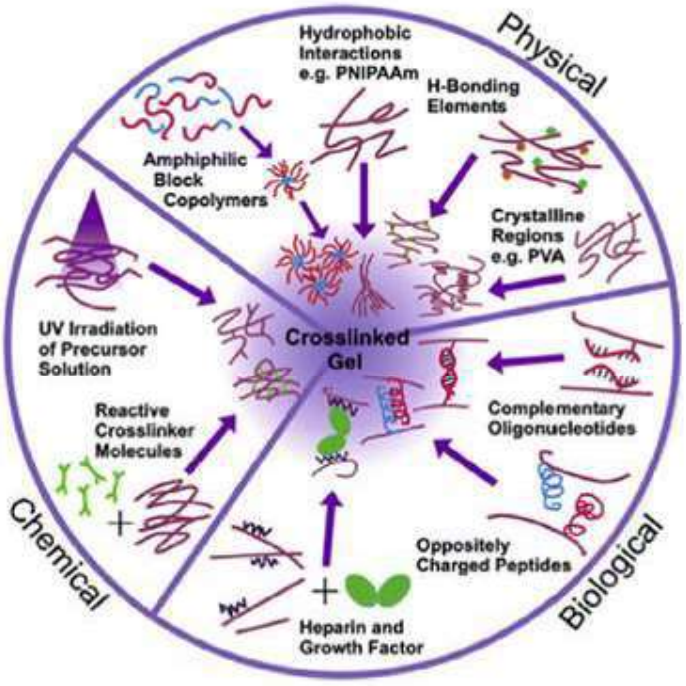

Fig. 1: Summary of cross-linking types [29].

Physical hydrogels network is based on chain entanglements formed by interactions such as ionic, hydrophobic or hydrogen bonds. Physical gels are reversible when changed the environmental

conditions such as pH, temperature, the ionic strength of solution, salt concentration, electric field or amount of solvent. Chemical gel is the network of covalent bonds with irreversible character and biological cross-linking creates compounds in the biological system [5, 6, 7].

2.1.1 Amphiphilic Block Copolymers

Copolymerization is used to modify the properties of prepared materials to meet specific needs, for example to reduce crystallinity, control wetting properties or to improve solubility. It is a way of promoting physical and chemical properties [8].

One of the copolymers' types is a block copolymer. It is a linear arrangement of blocks with various monomer composition. Block copolymer comprises two or more homopolymer units linked by covalent bonds. Block copolymers with two or three blocks are called diblock copolymers and triblock copolymers (Fig. 2) [9].

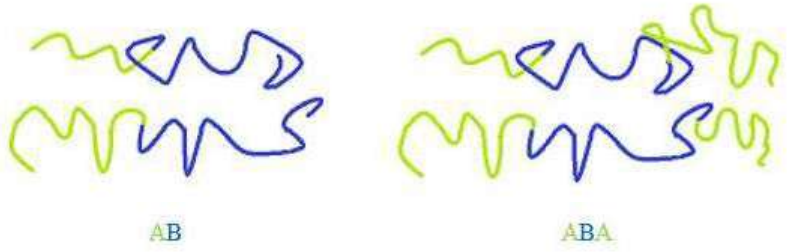

Fig. 2: Illustrative alignment of the blocks in the diblock and triblock copolymers.

The connection of two homopolymers with different properties (A, B) into the block copolymer brings new material that carry the properties of each homopolymer block. Amphiphiles have an affinity for both hydrophilic and hydrophobic environment, two opposite types of environments. The dual affinity is built by the covalent linkages of blocks with diverse chemical character, hydrophobic (A) and hydrophilic (B).

2.1.2 Micelles

Amphiphilic molecules self-organize at interfaces when dissolved in selective solvents. There is a typical feature of amphiphilic block copolymers, self-assembly. The earlier researches were aimed at organic solvents, but nowadays the studies are focused on the block copolymers' formations in aqueous solutions in order to use it in biological systems. Amphiphilic block copolymers are able to form various types of particles, spheres, capsules, polymersomes and micelles.

In aqueous environment, polymeric micelles are characteritic by a core–shell architecture, where the hydrophobic core is segregated from the aqueous area by a hydrophilic shell. The hydrophobic blocks of amphiphilic molecules are pointed away from water in order to achieve a state of minimum energy and the hydrophilic blocks surround hydrophobic core to create protective shell. Diblock copolymers self-assemble into star-like micelles and triblock copolymers form flower-like micelles (Fig. 3). At higher concentrations a dense network is formed.

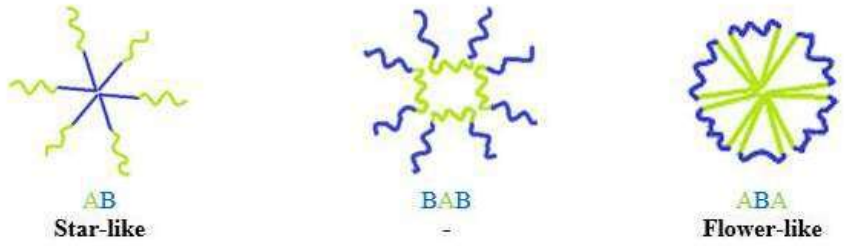

Fig. 3: Schematic representation of the aggregation for amphiphilic diblock and triblock copolymers.

Micelles are classified as an amphiphilic colloids, not a solid particles and their diameters ranging from 10 to 100 nm. There are three types of stimuli that micelles respond to. In according to stimuli-responsitivity, micelles are distinguished between pH sensitive, thermosensitive and sensitive to electric field [10 - 13]. The rest of diploma thesis will be concerned with thermosensitive micelles.

2.1.3 Thermosensitive Amphiphilic Block Copolymers

The most of amphiphilic block copolymers forms micelles in water, however only a small part of copolymers gels upon heating. This small part of copolymers includes copolymers with appropriate balance of hydrophilicity and hydrophobicity. The system of thermosensitive amphiphilic block copolymers undergoes a phase transition from liquid to solid phase. The transition is called sol-gel transition and it is defined by critical gel concentration and critical gel temperature. The terms critical gel concentration (CGC) and critical gel temperature (CGT) mean the lowest concentration and temperature at which the polymer system forms the gel. If the copolymer concentration is over its CGT and CGC, it will undergo the sol-gel transition.

During the sol-gel transition, the separation of hydrophobic and hydrophilic blocks occurs, but it cannot lead to complete segregation, because the blocks are chemically bonded via ester links. The blocks A and B form domains, as shown in the figure 4.

The thermogel has the structure of micelle network. The advantage of ABA type micelles is the fact that it can create dense network with higher strength and elasticity than BAB type in aqueous solution. ABA micelles are connected to each other and it leads to form more junctions located equally.

The gelation ability is influenced by many molecular structural parameters such as block length, the ratio and the sequence of homopolymers or the modification of the copolymer by additives. The thermogelling polymers have huge potential as injectable hydrogels in biomedicine with sustained release of drugs in the field of tissue engineering [10, 14, 15].

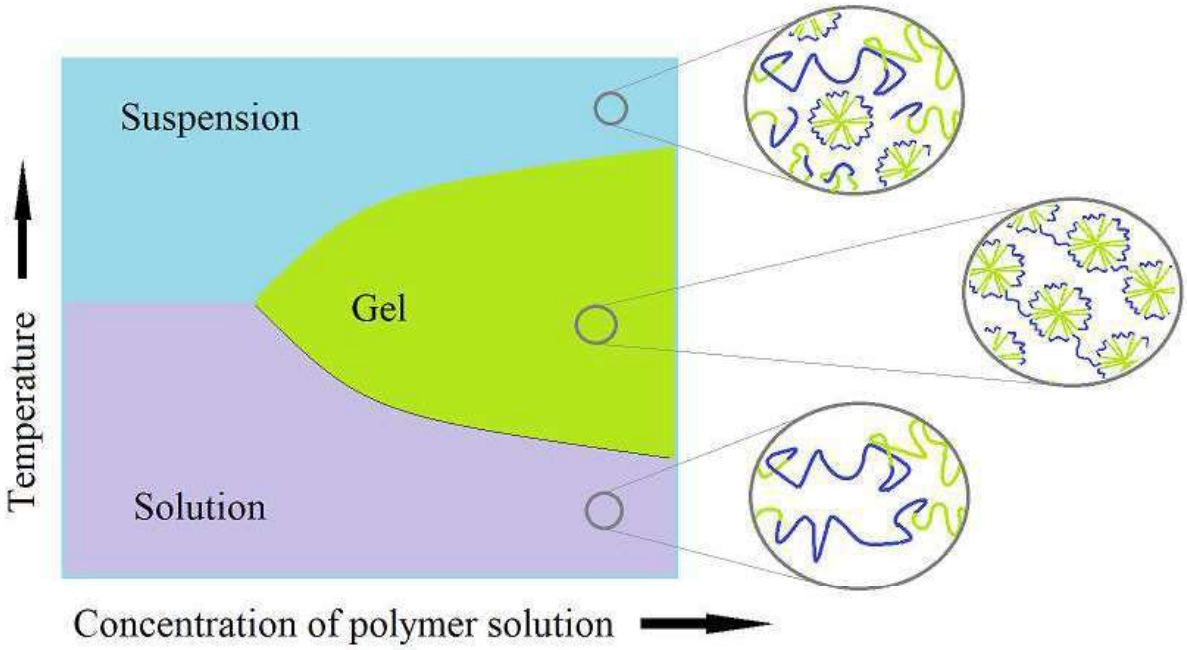

Fig. 4: Schematic sol-gel transition of thermosensitive amphiphilic copolymer.

2.1.4 Hydrolytic Degradation

For successful development of polymeric systems is important to understand the degradation mechanism of polymers. Degradation is the process resulting in molecular weight reduction by chain scission. In general, degradation can be caused by different agents that differentiate it into few types including hydrolytic, thermal, oxidative degradation etc.

Materials suitable for application in regenerative medicine such as matrices for drug delivery, scaffolds for tissue engineering or resorbable implants in orthopedic surgery have to degrade to non-toxic products in the biological environment of the living body. Described materials are called biomaterials and underlie the biodegradation. The most of biodegradable polymers contains hydrolysable bonds which enable chemical degradation via hydrolysis.

There are two well-known mechanisms of polymer hydrolitic degradation, bulk and surface erosion (Fig. 5). The way that polymer erodes depends on the diffusivity of water inside the bulk and on the degradation rate of the polymer's functional groups. The only difference between them is in the degradation kinetics. If polymer degrades slowlier than water penetrates into the polymer bulk it is called bulk (homogeneous) erosion. If polymer degrades faster than water penetrates into the polymer bulk it is called surface (heterogeneous) erosion.

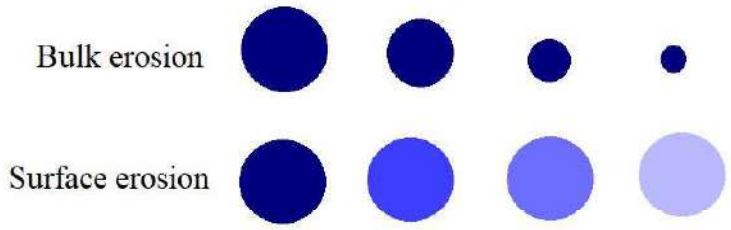

Fig. 5: Schematic illustration of bulk and surface erosion.

In order to design polymeric device with long-term life, there is a necessity to use surface-eroding polymers such as polyanhydrides or polyesters. Erosion rate for ideal surface-eroding polymer is constant and proportional to surface area. Bulk-eroding polymers do not have constant erosion rate.

The polymer degradation is influenced by many factors, such as chain character, crystallinity, morphology, copolymer ratio, distribution of molecular weight or substances with low molecular weight [7].

2.2 PLGA-PEG-PLGA Triblock Thermosensitive Copolymer

Biodegradable polymers, especially linear aliphatic polyesters derived from lactic acid (LA), glycolic acid (GA) and their copolymers have noticed a huge interest in the field of tissue engineering as the drug carriers with controlled release for different medical applications such as surgical implantation or wound treatment [16].

2.2.1 PLGA Copolymer

Polyester PLGA is a copolymer of poly(lactic acid) with abbreviation PLA and poly(glycolic acid), PGA. Poly(lactic acid) creates two enantiomeric forms: poly(D-lactic acid), PDLA, and poly(L-lactic acid), PLLA. The physico-chemical properties of optically active PDLA and PLLA are nearly the same. In general, the polymer PLA can be made in highly crystalline form (PLLA) or completely amorphous (PDLA) due to disordered polymer chains. In contrast to PLA, PGA has not got the methyl side groups and shows highly crystalline structure [1, 17].

Generally, PLGA is an abbreviation for poly(D,L-lactic-co-glycolic acid) where D- and L-lactic acid forms are in equal ratio (Fig. 6). The copolymeration is proceeded in order to improve properties of final material. PLGA can be processed into almost any shape or size and it can encapsulate molecules of any size. It is soluble in wide range of common solvents including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate. In water, PLGA biodegrades by hydrolysis of its ester linkages. The presence of methyl side groups in PLA makes it more hydrophobic than PGA and that is why lactide rich PLGA copolymer is less hydrophilic, absorbs less water and consequently degrades more slowly.

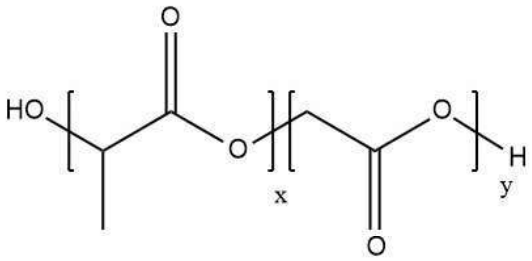

Fig. 6: Structure of poly(D,L-lactic-co-glycolic) acid (x is the number of lactic acid units and y is the number of glycolic acid units).

Due to the hydrolysis of PLGA, parameters that are typically considered as a constant factors of a solid formulation can be changed by the time, such as the glass transition temperature (T_g), moisture content and molecular weight. The changes in PLGA properties during the biodegradation of copolymer influence the release and the degradation rates of incorporated drug molecules. PLGA physical properties depend on multiple factors including the initial molecular weight, the ratio of lactide to glycolide, the size of the device, exposure to water, surface shape and storage temperature.

The degree of crystallinity of the PLGA directly influences properties like mechanical strength, swelling behavior, capacity to undergo hydrolysis and subsequently biodegradation rate of the polymer.

The application of the biodegradable copolymer PLGA represents great potential as a delivery vehicles for drugs, proteins or various other macromolecules like DNA, RNA or peptides and as the scaffolds for tissue engineering. PLGA copolymers are involved in a family of Food and Drug Administration (FDA) approved biodegradable polymers that are physically strong and highly biocompatible. PLGA is the most popular among the various available biodegradable polymers because of its long clinical experience, appropriate degradation characteristics and possibilities for controlled drug delivery.

Additionally, it is possible to modify the physical properties of the copolymer and to conjugate drugs by regulating the suitable parameters such as molecular weight, ratio of lactide to glycolide and drug concentration to achieve a needed dosage and release interval depending on the drug type [1].

2.2.2 Poly(ethylene glycol)

The biodegradable polyesters are strongly hydrophobic and this has led to some limitations in practical drug formulations. To eliminate them it is necessary to add the hydrophilic property to prepared copolymer. The way of adding hydrophilic character is the incorporation of hydrophilic poly(ethylene glycol), PEG, into the biodegradable polyesters (Fig. 7).

PEG is a non-toxic, water-soluble polymer with provable biocompatibility. Block copolymers consisting of a hydrophobic polyester segment and a hydrophilic PEG segment have achieved large attention due to their biodegradability, biocompatibility, and tailor-made properties.

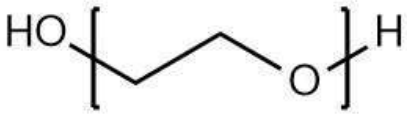

Fig. 7: Structure of poly(ethylene glycol).

The biodegradation rate and the hydrophilicity of block copolymers can be modulated by changing the ratio of its hydrophilic and hydrophobic segments. Usually, PLGA-PEG block copolymers show quite different properties when compared to each constituting block of copolymer. For this reason, PLGA-PEG block copolymers became a new family of biomaterials with their own unique properties, such as phase separation, crystallinity, water-solubility, and biodegradability.

Various kinds of block copolymers can be classified in according to their sequence of blocks, structure and thus different behaviour. The classes are, for example, an AB diblock, ABA or BAB triblock and multi-block, in which A is a hydrophobic block made up of biodegradable polyester and B is a hydrophilic PEG block [17].

2.2.3 Properties of PLGA-PEG-PLGA Thermosensitive Copolymer

The advancement in polymer science and engineering has designed new polymers for well-controlled delivery of therapeutic drugs. The smart polymers (stimuli-sensitive polymers) which carry active responsiveness to environmental signals and changes the physico-chemical properties as designed.

Physical (temperature, ultrasound, light, electricity, mechanical stress), chemical (pH, ionic strength), and biological signals (enzymes, biomolecules) have been used as triggering stimuli [4]. Triblock copolymers of both ABA and BAB type can act as a thermogel with an A-block covalently coupled with a B-block via ester link [17].

2.2.3.1 Amphifilic Character and Micellar Behaviour

This temperature sensitive copolymer, PLGA-PEG-PLGA, is an amphiphilic block copolymer. An amphiphilicity means consolidation of chemical behaviour, both hydrophilic and hydrophobic parts [18]. They are composed of hydrophobic PLGA segments and hydrophilic PEG segments.

Amphiphilic PLGA-PEG block copolymers form micelles composed of a hydrophobic PLGA core and hydrophilic PEG shell in water, as shown in the figure 3. Hydrophobic blocks are segregated from the aqueous environment to form an inner core surrounded by a layer of hydrophilic segments. Block copolymer micelles are soluble in water and create biocompatible formations with improved efficiency of delivering hydrophobic drugs.

These micelle-forming block copolymers can provide high concentration of hydrophobic drugs with increased drug stability in an aqueous milieu above the solubility limit of the drug. The ability of polymeric micelles to target certain cells, for example tumors, can also decrease the required dosage. The size and morphology of block copolymer micelles can be easily changed by conforming the chemical composition, total molecular weight, and ratio of the block lengths [17].

2.2.3.2 Temperature Sensitivity

Temperature sensitivity is based on the balance between hydrophobic and hydrophilic segments, which can be either monomer units or polymer blocks. In aqueous environment, inter- and intra-molecular interactions between hydrophobic segments results in polymer chain aggregation of physical cross-linking [4]. For instance, the PLGA-PEG-PLGA copolymer is a free flowing solution at room temperature and can form a viscous gel at body temperature. The hydrophobic PLGA segments form crosslinks and the hydrophilic PEG segments allow the copolymer molecules to stay in solution.

At lower temperatures, hydrogen bonds between hydrophilic PEG segments and water molecules causes the creation of the aqueous solution. The result is their dissolution in water. As the temperature increases, the hydrogen bonds become weaker, hydrophobic forces among the PLGA segments are stronger. This change leads to sol-gel transition (Fig. 8). Due to their sharp sol-gel transition, this copolymer has a huge potential for applying it as an injectable drug delivery system [16, 17].

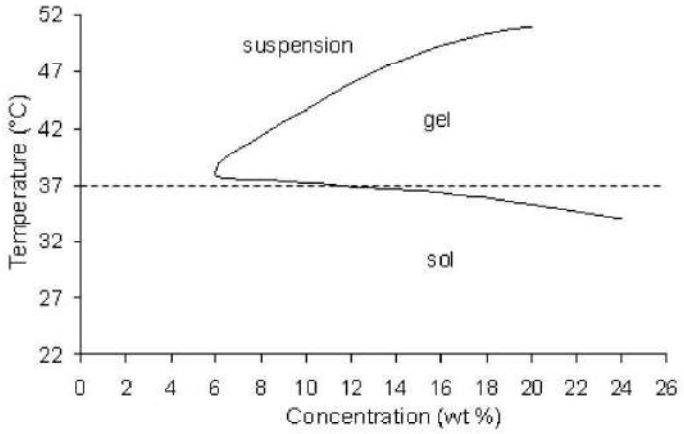

Fig. 8: Phase diagram of PLGA-PEG-PLGA copolymer with ratio PLGA/PEG 2 and LA/GA 3 in water [16].

The solution of PLGA-PEG-PLGA copolymer can be injected into the body and subsequently forms a soft gel at body temperature. Thermosensitive copolymers are very attractive for protein drug delivery, because no organic solvent is used during the linking of drug. However, they can also solubilize hydrophobic drugs and they are an excellent option for water insoluble drugs [4].

2.2.3.3 Water Solubility

The chemical composition and the molecular weight of block copolymers decide on their water solubility and degradation kinetics. Copolymers with low molecular weight or with composition of shorter hydrophobic blocks are soluble in water, whereas high molecular weight copolymers and copolymers with longer hydrophobic blocks are not soluble but swell in water.

Block copolymers consisting of hydrophilic and hydrophobic blocks are able to form physical crosslinking in an aqueous environment through hydrophobic interaction, crystalline domains or chain entanglement. Physical associations keep hydrophobic domains together and maintain the polymer network stable in water. Although physical associations are reversible and weaker than chemical crosslinks. They allow thermal processing and the final polymer gel often show elastic or viscoelastic properties. Biodegradable physical hydrogels offer an alternative opportunity in designing drug delivery systems [17].

2.2.3.4 Biodegradability

Useful strategy for modifying the physico-chemical and biological properties of hydrophobic biodegradable PLGA copolymer is the way of incorporating the hydrophilic PEG segment. It is known that low molecular weight PEG is easily excreted in human organism. Prepared block copolymer PLGA-PEG-PLGA is biodegradable. It means that PLGA-PEG-PLGA undergoes the hydrolysis

at physiological conditions to lactic and glycolic acids as a biocompatible and toxicologically safe by-products which are consequently eliminated via Krebs cycle to non-toxic products water and carbone dioxide.

In general, the degradation time is shorter for low molecular weight copolymers, more hydrophilic copolymers, more amorphous copolymers and copolymers with higher content of glycolide. Therefore, low molecular weight copolymer of lactide and glycolide degrades relatively rapidly, whereas the high molecular weight homopolymer, PLA or PGA degrades slowlier at identical conditions [2, 17].

2.2.3.5 Biocompatibility

Biocompatibility, the ability to be in contact with a living system without producing unfavourable effects, is clearly important. Although it is important to note that biocompatibility is not an intrinsic property of a material, but depends on the biological environment and the tolerability that exists with respect to specific drug-polymer-tissue interactions [1, 19].

2.2.3.6 Application

Various types of drug formulations such as particles, hydrogels, micelles, and injectable delivery systems have been developed using PLGA-PEG block copolymers to deliver hydrophobic drugs as well as hydrophilic drugs.

Aqueous solutions of ABA type triblock copolymers are well known to have thermoreversible sol-gel transitions, forming in situ hydrogels. Figure 8 represents the thermoreversible sol-gel transition of the triblock copolymer PLGA-PEG-PLGA. The system can be loaded with drugs in aqueous phase at low temperature, under the critical gelation temperature, where it forms a sol. Following injection increases the temperature to 37°C above critical gelation temperature and it makes the injected sol to a gel that can release loaded drug. For instance, injected copolymer can release loaded bone growth factors at the site of bone injury (Fig. 9).

Thermosensitive hydrogels have recently attracted large attention due to the simplicity of drug application and biocompatibility with biological systems. Pharmaceutical and biomedical applications of the block copolymers include solubilization of low molecular weight hydrophobic drugs, controlled release of labile biomacromolecules for example proteins and genes, cell immobilization and tissue engineering [17, 20].

Fig. 9: Illustrative options of application thermosensitive amphiphilic copolymer [21, 22].

2.3 Chemical Functionalization of Thermosensitive Copolymers

Recently, new types of modified multiblock copolymers have been developed for drug delivery because of their versatile character.

2.3.1 Succinic Anhydride

Succinic anhydride is linearly linked in PLGA–PEG–SA–PEG–PLGA multiblock copolymer in order to decrease molecular weight of prepared multiblock copolymer to improve biocompatibility, biodegradability and drug efficacy of the biomaterial (Fig. 10).

Fig. 10: Chemical structure of succinic anhydride.

PLGA–PEG–SA–PEG–PLGA multiblock copolymer (Fig. 11) is synthesized in two steps using direct polycondensation. In the first step, polyethylene glycol succinate (SAP) is prepared by direct melt polycondensation of succinic anhydride and PEG. During this process, PEG first undergoes thermal depolymerization to give low molecular weight and subsequently reacts with succinic anhydride to give white SAP. Therefore, achieved molecular weight is lower than the expected value.

In the second step, multi-block copolymer is synthesized by direct melting polycondensation of PLGA and SAP under the nitrogen atmosphere. The molecular weight of the copolymer depends on the amount of SAP in the reaction mixture. When the amount of SAP is increased in the reaction mixture, it causes depolymerization of PEG and the molecular weight of synthesized multiblock copolymer is rapidly decreased.

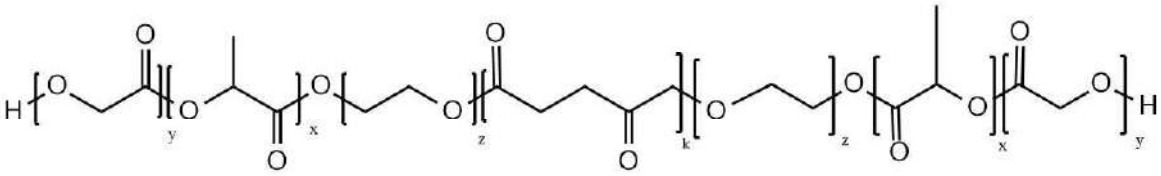

Fig. 11: Multiblock copolymer PLGA–PEG–SA–PEG–PLGA; x is number of PLA subunits, y is the number of PGA units, z is the number of PEG units and k is the number of SAP units [23].

Rifampicin (RIF) is an anti-tuberculostatic agent. The treatment of tuberculosis is improved by formulating this drug into the controlled release system such as PLGA–PEG multiblock copolymer. Modified multiblock copolymer PLGA–PEG–SA–PEG–PLGA is used to prepare RIF loaded polymer [23].

2.3.2 Folic Acid

Commercial pharmaceutical devices include PLGA-based domains such as particles, liposomes, capsules, micelles or spheres. However, the body considers them as a foreign particles. The reticulo-endothelial system easily eliminates these domains from the blood stream and takes them up in the liver.

To address the limitations, hydrophilic block of copolymer, poly(ethylene glycol) (PEG), is generally incorporated into the structure of copolymer or layered on the surface of the domains. Targeted drug release system can further improve the bioavailability of drugs. Ligand-modified carriers of drug delivery systems are paid much attention for their site-specific targeting capacity.

Folic acid (FA), as a targeting ligand, is extensively used to immobilize on the surface of polymeric carriers to deliver drugs into cells via receptor-mediated endocytosis (Fig. 12) [18]. Folic acid decorated

spheres, liposomes or micelles of biodegradable polymers such as PEG-PLA, PLGA-PEG or PEG-PCL (polycaprolactone) increase the cellular uptake and cell cytotoxicity of the formulated anticancer drugs [24]. The introduction of folic acid on the surface of PLGA-PEG copolymers is the achievement of active targeting to the tumor cells and the improvement of the cellular uptake of the drugs in tumors [25].

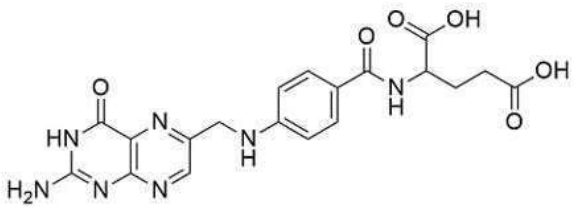

Fig. 12: Chemical structure of folic acid [24].

The amphiphilic block copolymer PLGA-PEG-PLGA with the FA-end molecule represents a new group of efficient drug carrier that appears to be suitable for controlled and targeted release in a wide range of drugs. FA-PLGA-PEG-PLGA copolymer is effective for encapsulating the hydrophobic drug, has high encapsulation efficiency and a good stability in the blood stream (Fig. 13).

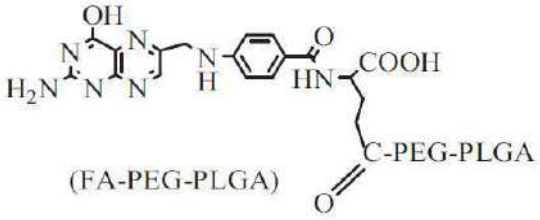

Fig. 13: Chemical structure of PLGA-PEG modified by FA [24].

Colorectal cancer is the third most common cancer worldwide. Capecitabine (CAP), a prodrug which can convert to the active metabolite via enzyme pathway in the target tissue, has been improved to be the most successful approach for the treatment of early and advanced colorectal cancer. Polymeric carriers with PLGA segments with terminally conjugated CAP located in the core and PEG segments with terminally conjugated FA creates the shell oriented outside toward aqueous milieu. CAP is widely used alone or in combination during the treatment of CRC. For example, CAP/oxaliplatin is highly effective for treating colorectal cancer [24].

Vincristine sulfate (VCR), which is a cell cycle-specific anticancer agent, is widely used in cancer chemotherapy. Its cytotoxic activity is based on the ability to inhibit microtubule and alter microtubule's structure and function to stop cellular division in metaphase [25].

2.3.3 Itaconic Anhydride

Thermosensitive copolymer PLGA-PEG-PLGA has been modified by itaconic anhydride (ITA) in order to functionalize both ends of the copolymer (Fig. 14).

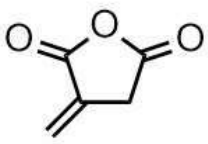

Fig. 14: Chemical structure of itaconic anhydride.

PLGA-PEG-PLGA copolymer functionalized with itaconic anhydride (ITA) involves reactive double bonds and functional carboxylic acid groups at the ends of copolymer resulting in preparation of ITA/PLGA-PEG-PLGA/ITA macromonomers (Fig. 15) [16].

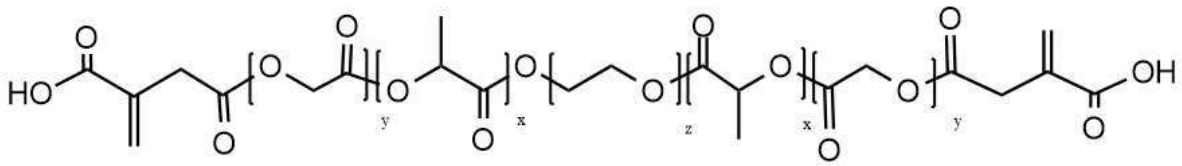

Fig. 15: Chemical structure of ITA/PLGA-PEG-PLGA/ITA triblock copolymer.

Itaconic anhydride (ITA) introduces carboxylic groups for bioactive compounds bonding and double bonds suitable for chemical cross-linking at the ends of the copolymer. The influence of the presence of carboxylic groups at the end of ITA/PLGA-PEGPLGA/ITA on a sol-gel phase transition in comparison to unmodified PLGA-PEG-PLGA has positive effect, it reduces the gel temperature range.

The ITA/PLGA-PEG-PLGA/ITA macromonomers are light-, temperature-, and pH-sensitive and can be cross-linked either chemically by covalent bonding through the double bonds, for example via photopolymerisation, and physically through hydrogen bonds or ionic interactions, in order to produce a new functionalised hydrogel network.

Additionally, functional carboxyl groups can be used as coupling sites to increase the hydrogel's biocompatibility, bioinductivity, adhesion or other physical properties and that is the reason why it may be tailored for certain types of biomedical applications such as injectable polymer drug delivery systems, tissue implants or resorbable bone adhesives.

ITA can be obtained from renewable resources, either by distillation of citric acid or by pyrolysis of itaconic acid which can be prepared by the large-scale fermentation of polysacharides with Aspergilus terreus. It is known that itaconic anhydride passes on to itaconic acid by hydration in the presence of moisture, which might evoke undesired side reactions for example acidolysis, esterolysis, hydrolysis and this reactions inhibit polymer modification. Sublimation of ITA under reduced pressure removes water and enables to itaconic acid to turn back to anhydride by dehydration.

It is known that ITA undergoes degradation under physiological conditions to non-toxic products. Acetate, lactate, and carbon dioxide were observed as the main degradation products. Based on the thermal analyses, ITA improves thermal properties of original PLGA-PEG-PLGA copolymer, increases thermal stability of PLGA ester bonds by ITA linking. ITA/PLGA-PEG-PLGA/ITA copolymer is more stable and starts degradation later. Final functionalized macromonomer has became suitable biomaterial in the field of tissue engineering [2, 20].

2.4 Linker

The advancement of tissue engineering in the new materials development offers many options in the field of regenerative medicine. Smart polymers system (stimuli-sensitive polymers answering to environmental signals by designed process) enables the solubilization of low molecular weight hydrophobic drugs, their accurate delivery and controlled release to required place in the body. Controlled drug release is aimed at targeted cells with high local concentration for a long time but it does not influence circumambient healthy cells. It results in the maximum of the therapeutic efficiency and in the minimum of undesirable side effects.

There is one component responsible for this function called linker. Linker is supposed to be degraded when recognising the environmental signal. Active drug is generated by continuous or specific degradation of linker, conjugative chemical between drug and polymer.

Another important function of linker is to maintain the biological activity of attached peptide (drug) by elongating of the polymeric chain. Proteins' specific biological activity is based on its conformation and structure. If protein was conjugated to polymer stiffly, it could lose the bioactive character by restrainting of the chain movement. Additionally, linkers with aromatic nucleus can stabilize biological active compounds.

Progress in the treatment of certain disease usually depends on multiple biological components such as growth factors, enzymes, antibiotics or leukocytes. To solve this issue, programmed drug release

system is an excellent solution to deliver multiple drugs. In this system, multiple drug components are required to be released sequentially [4].

2.4.1 Click Chemistry

In organic synthesis, click chemistry is a mild type of biocompatible small molecule reactions used in bioactive compound conjugation. Click chemistry describes a way of generating products that follow examples in nature.

In general, the participants of click reactions are both reporter molecule (linker) with sensitive polymer and the bioactive molecule. Click reactions run in one pot, hence they are not disturbed by water or other organic solvents, produce minimal and non-toxic byproducts, generate stable products and proceed quickly to high yields. These qualities make the concept of click chemistry suitable for synthesis of biocompatible materials [26].

2.4.2 Dopamine

Dopamine (abbreviation DOPA) is an organic chemical compound including aromatic nucleus, amino and hydroxyl groups. Dopamine is very important compound in human physiology, it performs as a neurotransmitter and also as a hormone.

2.4.2.1 The Structure of Dopamine

The molecule of dopamine, 4-(2-aminoethyl)benzene-1,2-diol, is composed of a catechol structure and phenethylamine. Catechol structure consists of benzene nucleus with two hydroxyl side groups and one amino group connected to aromatic nucleus via ethyl chain representing the phenethylamine system (Fig. 16).

Dopamine is the simpliest catecholamine, a family that also includes another neurotransmitters: norepinephrine and epinephrine. The presence of aromatic nucleus with attached amino group makes it more specific, this compounds are active drugs that influence the function of certain organs in human body [27].

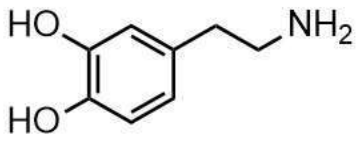

Fig. 16: Chemical structure of dopamine.

2.4.2.2 Chemical Properties

Acid-base properties of dopamine are clear. Dopamine, as other amines, is an organic base. In acidic environment dopamine occurs in protonated form. The protonated form is relatively stable and highly water-soluble, but it can become oxidized easily if exposed to oxygen, air humidity or other oxidants. On the other hand, in base environment it is less water-soluble and more reactive, the highest reactivity of dopamine varies above pH value 8.5.

Dopamine is sourced for the chemical or pharmaceutical usage as dopamine hydrochloride because the protonated form disposes of the increased stability and water-solubility. The hydrochloride salt is created when dopamine is combined with hydrochloric acid. In dry form, dopamine hydrochloride is a fine white powder [28, 29].

2.4.2.3 Adhesivity of Dopamine

Dopamine shows very interesting feature, it is responsible for the adhesion in the water milieu. This is the fact found by studying marine mussels and aquatic organisms which can adhere to any surfaces under the waterline.

The mechanism of adhesion is based on the transformation of the surface character. The substrate with hydrophobic surface was immersed into a water solution containing dopamine, underwater bioadhesive compound. The hydrophobic surface was transformed into the surface with hydrophilic properties. The immersed surface remained hydrophobic but it adhered in water environment. It is obvious that polydopamine formed by oxidative self-polymerization of dopamine coated hydrophobic surface and hence it modified surface character. This conversion of surface features supports the fact that polydopamine can control surface hydrophobicity.

Nowadays well known surface chemical method, called polydopamine coating, published Lee, Messersmith, and co-workers. They identified dopamine that contains both side chain functionalities of DOPA and L-lysine, essencial amino acid. Polydopamine can modify a wide variety of material surfaces, such as noble metals, metal oxides, bioceramics, and synthetic polymers with protein immobilization, metallization, biomineralization, and cell adhesion [26, 30].

The posibilities of application for effective underwater adhesives are opened, for example attaching sensors, beacons, stopping watery leaks or in medicine area, repairing wet living tissues. Synthetic adhesives developed for dry applications could not be used on wet surfaces, or even under the water. Despite of bulk cohesive strength much greater than natural underwater adhesives, they fail because of poor interfacial adhesion in the presence of water [31, 32].

2.4.2.4 Biological Function of Dopamine

Dopamine is a hormone and neurotransmitter occurring in a wide variety of animals including both vertebrates and invertebrates. Neurotransmitter, a chemical messenger, helps in the transmission of signals in the brain and other vital areas. Dopamine is a neurohormone, too, released by the hypothalamus.

Its main function as a hormone is to inhibit the release of prolactin. Dopamine produced by neurons in the arcuate nucleus of the hypothalamus is released in the hypothalamo-hypophysial blood vessels. This acts on the lactotrope cells that produce prolactin and that is why dopamine is called prolactin-inhibiting hormone or prolactostatin.

Dopamine has many functions in the brain, including important roles in behavior and cognition, motor activity, motivation and reward, sleep, mood, attention and learning. A part of the brain called the basal ganglia regulates movement. When there is a deficiency of dopamine in the brain, movements may become delayed and uncoordinated. On the flip side, if there is an excess of dopamine, the brain causes the body to make movements such as repetitive tics.

The amount of dopamine influences memory, attention and also cognition. Levels of dopamine in the brain, especially the prefrontal cortex, helps in focus and improves working memory. Deficiency of this vital chemical is the cause of several disease conditions. Parkinson's disease and drug addiction are some of the examples of problems associated with abnormal dopamine levels.

Parkinson's disease is a degenerative state caused by decreased number of dopamine-releasing neurons in the area of the midbrain called the substantia nigra. Dopamine plays a role in pain processing in multiple levels of the central nervous system. Also some features of negative schizophrenia are thought to be related to a low dopaminergic state in certain areas of the brain.

Restless legs syndrome and attention deficit hyperactivity disorder (ADHD) are associated with decreased dopamine activity. Dopaminergic stimulants can be addictive in high doses, but some are used at lower doses to treat ADHD [33].

Cocaine and amphetamines inhibit the re-uptake of dopamine. They are dopamine transporter blocker that competitively inhibits dopamine uptake to increase the presence of dopamine. By increasing of the dopamine amount and accumulation it leads to intensive pleasurable feelings and consequently to addiction.

2.4.2.5 Drugs

Dopamine is FDA approved and additionally, dopamine is needed drug in brain disease. It influences the sympathetic nervous system and increases blood pressure and heart rate by raising its contractility. This medication is useful in the treatment of heart failure or shock, especially for newborn babies.

In general, linker has to be biocompatible, biodegradable and should be versatile to ensure various efficiency. Described properties of dopamine prefer its to the usage as a linker and, according to certain point of view, as a biologically active compound, too [34].

2.5 Bioactive Substances

Described thermosensitive copolymer with its great properties has many uses in the field of medicine. However, the cellular response such as adhesion, growth or proliferation of cells to this copolymer is weak. To develop biodegradable copolymer with ideal responsitivity of cells is convenient to prepare mentioned copolymer modified by bioactive substances. There are different kinds of biologically active compounds. Mostly their chemical character is the same, they belong to the category of proteins and bring the beneficial properties to the polymeric device.

2.5.1 L-lysine

Lysine is a proteinogenous amino acid with abbreviation Lys or K. Chemically, it is the aliphatic α-amino acid with the structure including amino group (-NH_2), carboxylic functional group (-COOH) and the side chain lysyl $(CH_2)_4NH_2$ (Fig. 17). It is obvious that Lysine has base character as well as amino acids Arginine and Histidine.

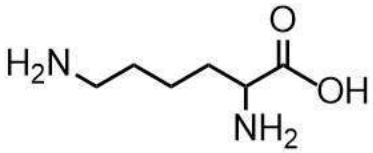

Fig. 17: Chemical structure of lysine.

In general, lysine is one of the essencial amino acids which means that the mammalian body cannot produce it and hence it must be received from the diet. Its metabolic way starts by transamination with α-ketoglutarate and leads to product acetyl-CoA. Plants and bacteria can synthesize it from aspartic acid.

L-lysine is a necessary basic component unit of all proteins in the mammalian body, plays a significant role in the absorption of calcium and its building in the bone tissue or the production of hormones, enzymes or antibodies. In the production of elastin and collagen is used derivated lysine synthesized by the enzyme lysyl oxidase. This process is important for the crosslinking that stabilizes the structure of collagen and elastin. L-lysine is industrially produced by fermentation with caprolactam and Corynebacterium glutamicum.

Lysine, as a essencial amino acid and thus important bioactive substance is necessary for healthy organism. The biodegradability of the copolymer affords it to make its purpose and degrade to components processed by the organism subseqentially.

Additionally, Lysine possess reactive functional groups that enable chemical modification in order to improve properties of used copolymer. From the past, L-lysine has been utilized in the area of gene delivery widely because of its characteristic structure with possible modifications. Incorporation of lysine into the polymer structure via linker allows reaction at the lysine's amine group that leads to a variety functionalities involved for example RGD (arginine-glycine-aspartic acid) peptide sequences or growth factors to increase interactions between polymer and cells.

In accordance with expectations, other biologically active proteins and pharmaceutical agents such growth factors or insuline can be attached in the same way to the polymeric material because of the same functional groups concerned in the structure [35-39].

2.5.2 Fibroblast Growth Factors

A growth factor is a biological compound occuring in the organism with the ability to regulate cellular processes and stimulate cellular growth, proliferation, differentiation or healing. Growth factors are signaling molecules that can attach to specific receptors located on the surface of target cells. There are many groups and categories of growth factors including fibroblast growth factors.

The group of FGF (fibroblast growth factors) includes twenty-three different FGF ligands from which twenty-two are human. They activate four types of receptors typical for this FGF group and their defining feature is binding to heparin released arround injury or tissue remodeling [40, 41].

FGF 2 or basic fibroblast growth factor (bFGF) (Fig. 18) is capable of regulation of many biological activities involving embryonic and cell development, tissue repairing or tumor growth. It is known that FGF2 protects the heart from injury connected with a heart attack and reducing tissue death [42, 43].

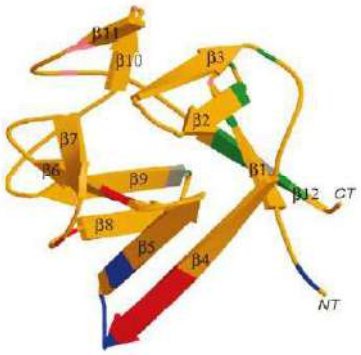

Fig. 18: 3D structural scheme of FGF 2 [42].

2.5.3 Insulin

Insulin is a protein composed of fifty-one amino acids and it is produced by pancreatic cells that functionates as a main anabolic hormone in the body (Fig. 19). It controls the metabolism of polysacharides, fats and proteins by stimulating the absorption of glucose from the blood. Insulin also regulates the conversion of small molecules into big molecules in the cells. On the other hand, the catabolism is caused by low levels of insulin and the result could be reserving the body fat.

Insulin improves the balance of blood sugar level and maintains it in a appropriate range. When the level of blood sugar increases, the pancreatic cells produce more insulin. The disorder in secretion of insulin may lead to serious disease called diabetes mellitus or hyperglykemia. Patients under the treatment are forced to receive insulin in the injectable form.

Nowadays, insulin is one of the most common and necessary drugs. Its chemical structure enables the connection with thermosensitive copolmer to create injectable hydrogel with controlled release of protein - drug insulin in according to mentioned expectations [44, 45].

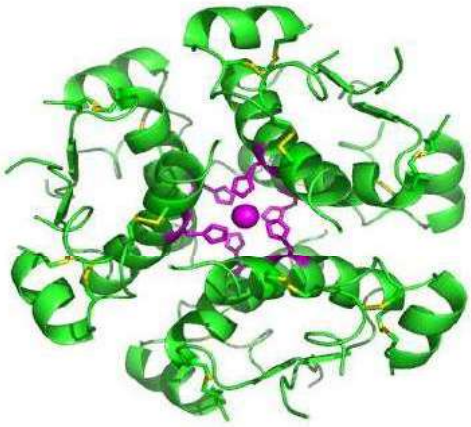

Fig. 19: 3D structural scheme of Insulin [45].

3 GOAL OF THE WORK

The diploma thesis is concerned with biodegradable PLGA-PEG-PLGA triblock copolymers. Prepared triblock copolymers are capable of forming hydrogel by physical cross-linking in consequence of their amphiphilic character. Used stimulus to form the hydrogel is increased temperature and observed sol-gel transition has a sharp curve at the body temperature (chapter 2.2.3.2). These materials have noticed significant interest in the field of medical sciences.

The ambition to improve PLGA-PEG-PLGA triblock copolymers studied by our group leads to functionalize and modify it. The functionalization by itaconic acid brings new opportunities to improve mentioned copolymers. Itaconic acid functionalizes both ends of the copolymer by reactive double bonds and carboxylic groups. The double bonds make the copolymer light-sensitive and they enable the chemical cross-linking. End-capped carboxylic groups offer the modification by biologically active compounds that enlarge the utilization of the material [16].

The modification affords the opportunity to create polymeric network enriched by compounds with biological activity needed for function in the medicine. The main reason of modification is to enhance the cellular response such as adhesion, growth or proliferation and advance the treatment of the damaged tissue.

The goal of the thesis is the synthesis and the characterization of thermosensitive biodegradable copolymers PLGA-PEG-PLGA functionalized by itaconic acid, modified by bioactive substances L-lysine and butylamine via linker dopamine.

Experimental part of diploma thesis includes following steps:

1. Synthesis of ITA functionalized copolymers ITA/PLGA-PEG-PLGA/ITA
2. Synthesis of DOPA functionalized copolymers DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA
3. Synthesis of ButA functionalized copolymers ButA-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-ButA or Lys functionalized copolymers Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys

4 EXPERIMENTAL PART

4.1 Chemicals

- 4-(Dimethylamino)pyridine (DMAP, > 99.0 %) was purchased from Fluka, Switzerland.
- Aceton (> 99.5 %) was purchased from Penta, s.r.o., Czech Republic.
- Butylamine (99.5 %) was purchased from Sigma – Aldrich, Germany.
- Calcium hydride (CaH_2, coarse granules, < 20 mm, reagent grade, 95.0 %) was purchased from Sigma – Aldrich, Germany.
- D,L-lactide (LA, > 99.9 %) was purchased from Polysciences Inc., Pennsylvania.
- Dicyclohexylcarbodiimide (DCC, 99.0 %) was purchased from Sigma – Aldrich, Germany.
- Diethyl ether (DEE, > 99.5 %) was purchased from Lach-Ner, s.r.o., Czech Republic.
- Dichloromethane (DCM, > 99.5 %) was purchased from Lach-Ner, s.r.o., Czech Republic.
- Dopamine hydrochloride (DOPA) was purchased from Sigma – Aldrich, Germany.
- Dow Corning® High Vacuum Silicone Grease was purchased from Sigma – Aldrich, Germany.
- Gaseous nitrogen (99.999 %) was purchased from SIAD Czech spol. s.r.o. and refined by drying column filled with molecular sieves and Cu catalyst.
- Glycolide (GA, > 99.9 %) was purchased from Polysciences Inc., Pennsylvania.
- Chloroform (> 99.5 %) was purchased from Lach-Ner, s.r.o., Czech Republic.
- Chloroform-d (99.8 Atom % D) for NMR spectroscopy was purchased from Sigma – Aldrich, Germany.
- Itaconic anhydride (ITA, > 98.0 %) was purchased from Acros Organics, Belgium.
- Liquid nitrogen was purchased from Linde Gas, a.s., Czech Republic.
- L-lysine (Lys, > 98.0 %) was purchased from Sigma – Aldrich, Germany.
- Methanol (MeOH, > 99.5 %) was purchased from Lach-Ner, s.r.o., Czech Republic.
- N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, >98.0 %) was purchased from Sigma – Aldrich, Germany.
- N,N- Dimethylformamide (DMF, > 99.5 %) was purchased from Penta, s.r.o., Czech Republic.
- N-Hydroxysuccinimide (NHS, > 97.0 %) was purchased from Sigma – Aldrich, Germany.
- Poly(ethylene glycol) (PEG) with M_n = 1500 g mol^{-1} was purchased from Sigma – Aldrich, Germany and degassed under vacuum for 3 hours.
- Sn(II)2-ethylhexanoate (Sn-octoate, 95.0 %) was purchased from Sigma – Aldrich, Germany.
- Tetrahydrofurane (THF, > 99.5 %) was purchased from Penta, s.r.o., Czech Republic.
- Toluene (> 99.5 %) was purchased from Lach-Ner, s.r.o., Czech Republic.
- Tris-(hydroxymethyl) aminomethane chlorhydrate (TRIS) was purchased from VWR Chemicals, Pensylvania.
- Ultrapure water (ultrapure water of Type 1 according to ISO 3696) was prepared on Millipore purification system (MilliQ Academic, Millipore, France)

4.2 Equipment

- 700 MHz NMR spectrometer Bruker AVANCE III (Bruker CO., Germany)
- Analytical scale Adventurer Pro AV64 (Ohaus GmbH, Switzerland)
- Drying oven (Ecocell 111, BMT a.s., Czech Republic)
- Elix® Essencial (Merck Millipore Co., Germany)
- FTIR-ATR, Tensor 27 (Bruker Co., Germany)
- Glass high vacuum line (Ceitec VUT)
- Glove box GP Concept (Jacomex, France)
- Christ Epsilon 2-10D LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany)
- Magnatic stirrer RCT basic (IKA, USA)
- Rheometer TA Intruments AR-2 (TA Instruments, USA)
- Vacuum drying oven (Vacucell 22, BMT a.s., Czech Republic)

4.3 Methods

The synthesis of copolymers was performed in the vacuum line and manifold (Fig. 20) through the use of Schlenk's technique under the nitrogen atmosphere.

Fig. 20: Vacuum line and manifold.

4.3.1 Synthesis of Functionalized Copolymers

The copolymer PLGA-PEG-PLGA (ABA) creates the basis for the application in the tissue engineering and its modification enables to meet another specific needs related to particular function. Copolymer PLGA-PEG-PLGA is prepared with PLGA/PEG weight ratio 2.5 and LA/GA molar ratio 3.

In comparison to the functionalized copolymer ITA/PLGA-PEG-PLGA/ITA (ABA/ITA) which has PLGA/PEG weight ratio 2.0 and LA/GA molar ratio 3.0 because functionalization by ITA decreases the gel temperature.

4.3.1.1 Synthesis of ITA/PLGA-PEG-PLGA/ITA Copolymer

The PLGA-PEG-PLGA triblock copolymer was synthesized by ring-opening polymerization in a bulk. A macromonomeric initiator, PEG (1500 g mol^{-1}, 2.1 mmol), was dehydrated under the vacuum at the temperature 130 °C for 3 hours. Then, after cooling the melt of PEG, LA (34.9 mmol) and GA (11.6 mmol) were added and degassed under the vacuum at room temperature for 1 hour.

Subsequently, the reactive mixture was heated up to temperature 130 °C and the catalyst, Sn(II)2-ethylhexanoate (Sn-octoate, 0.1 mmol), was injected into melted mixture. The dosage of the catalyst started the copolymerization lasting for 3 hours under the nitrogen atmosphere.

Following part of the synthesis was the functionalization of copolymer PLGA-PEG-PLGA by ITA (Fig. 21). Prepared PLGA-PEG-PLGA copolymer was left to cool down, later ITA (5.3 mmol) was added and degassed under vacuum for 1 hour. The temperature was increased at 110 °C and the reaction took 1 hour under the nitrogen atmosphere. The addition of ultra pure water terminated the reaction and began the process of purification (chapter 4.3.2.4). Final product had yellow colour. Particular samples are presented in the table 2.

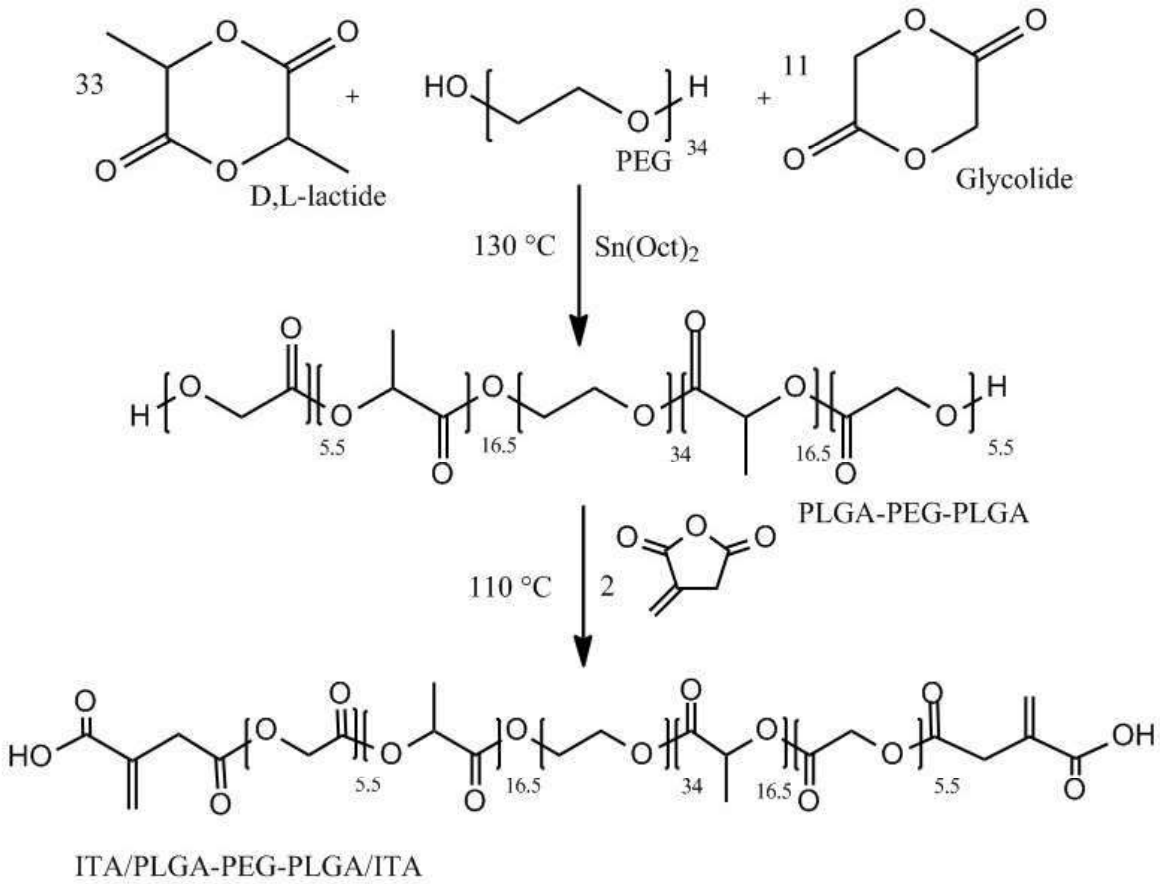

Fig. 21: Reaction scheme of ITA/PLGA-PEG-PLGA/ITA synthesis in a bulk.

4.3.2 Synthesis of Modified Copolymers

The synthesis of modified copolymers consisted of 3 steps. The first step was synthesis and functionalization of ITA/PLGA-PEG-PLGA/ITA copolymer mentioned above in chapter 4.3.1.1.

The second step was the modification by dopamine in order to extand the options of application. Dopamine has been considered as a bioactive compound, however it was intend for the function of linker and adhesive agent. If the dopamine modification was successful, the synthesis continued by the third step.

The third step included the linking of the bioactive compound. At the beginning butylamine was used instead of L-lysine because of its structural simplicity. Butylamine is linear aliphatic amine with any other functional group. Itaconic acid, dopamine, butylamine and L-lysine were used in abudance with ITA/PEG molar ratio 2.5 and DOPA/PEG, ButA/PEG, Lys/PEG molar ratio 2.0.

4.3.2.1 Dopamine Modification in Aqueous Solution

Aqueous environment is resemble to physiological environment in the living body because of the high water content. Biological compounds in the organism react under the mild conditions such as neutral pH or body temperature.

4.3.2.1.1 Without the Usage of Activating System

The aqueous solution of copolymer ITA/PLGA-PEG-PLGA/ITA (18.9 µmol, 0.7 mL) and dopamine (28.4 µmol, 0.3 mL) were prepared. The solution of dopamine was slowly dropped into the copolymer

solution under the stirring at laboratory temperature with the access of air for 2 hours with total volume 1 mL of ultrapure water (Fig. 22). The process of purification is described in chapter 4.3.2.4. Particular samples are presented in the table 2.

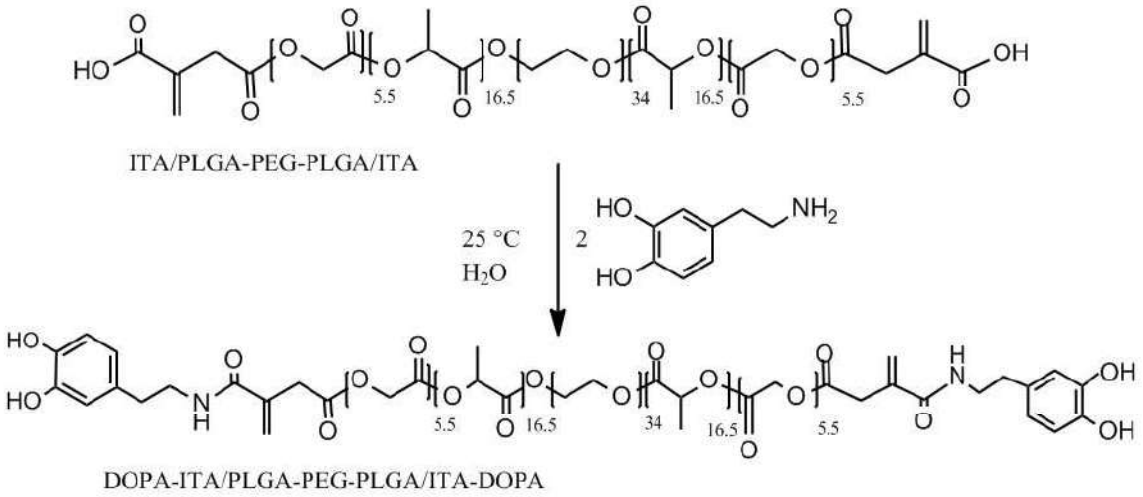

Fig. 22: Reaction scheme of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA synthesis.

4.3.2.1.2 In the Presence of Activating System EDC/NHS

The aqueous solution of copolymer ITA/PLGA-PEG-PLGA/ITA (18.9 µmol, 0.6 mL), dopamine (28.4 µmol, 0.3 mL) and N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride with N-Hydroxysuccinimide (EDC/NHS) (22.7 µmol, 11.4 µmol, 0.1 mL) were prepared.

Firstly, the solution of EDC/NHS dropped into the copolymer solution was left to activate the carboxyl functional groups of copolymer for 1 hour (Fig. 23).

Subsequently, the solution of dopamine was slowly dropped into the solution of copolymer with activated carboxyl groups. The reaction continued under the stirring at laboratory temperature for 2 hours with access of air, too (Fig. 24). Particular samples are presented in the table 2.

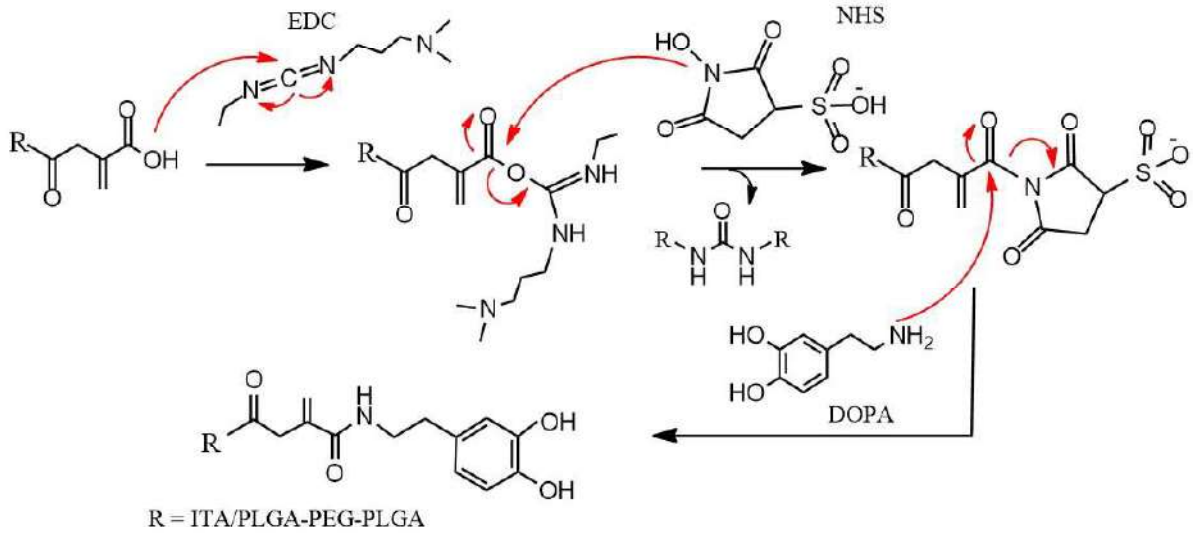

Fig. 23: Reaction mechanism of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA synthesis in the presence of activating system EDC/NHS.

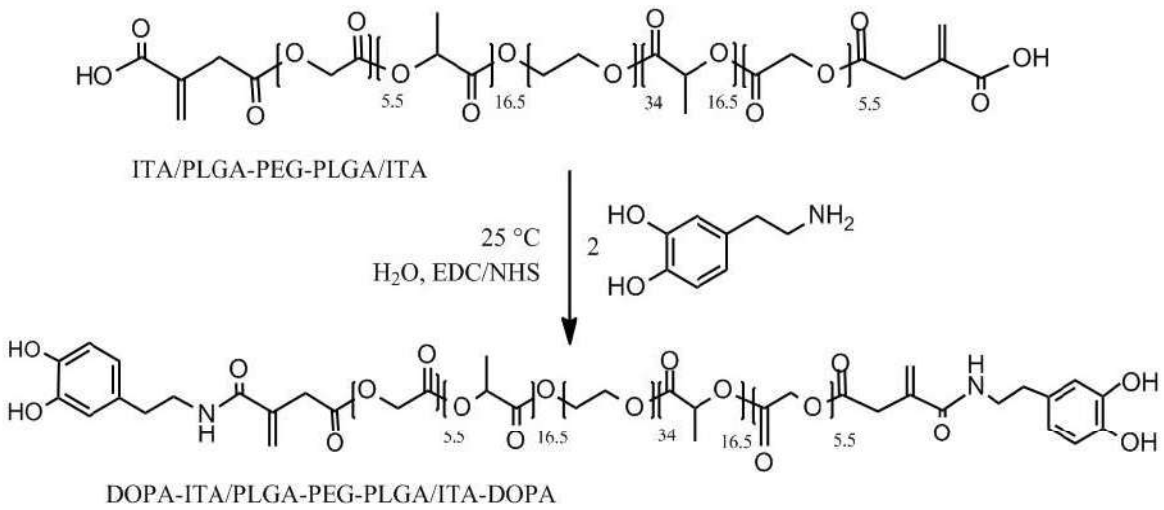

Fig. 24: Reaction scheme of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA synthesis in the presence of activating system EDC/NHS.

4.3.2.2 Dopamine and Butylamine Modification in Organic Solution

Before the choise of organic solvent, the test of dissolubility was accomplished (Fig. 25). Tested solvents and the results are presented in the table 1 from which emerges the decision to use N,N-Dimethylformamide (DMF). Prior to the usage, DMF solvent was distilled from calcium hydride.

Table 1: The dissolubility test overview.

Organic solvents	Chloroform	Acetone	Toluen	DMF	MeOH	DEE	DCM	THF
Label of sample	1	2	3	4	5	6	7	8
DOPA	×	×	×	✓	✓	×	×	×
ITA/ABA/ITA	-	-	-	✓	✓	-	-	-
Lys	-	-	-	×	✓	-	-	-

*Methanol (MeOH), Dichloromethan (DCM), Diethyl ether (DEE), Tetrahydrofurane (THF)

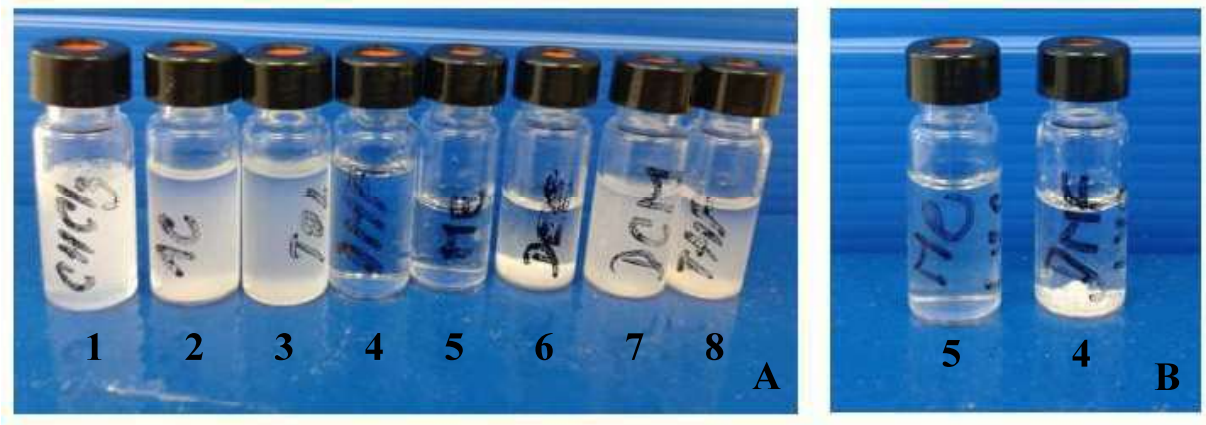

Fig. 25: The dissolubility test (A- the dissolubility test of DOPA, B- the dissolubility test of Lys).

4.3.2.2.1 In the Presence of Activating System DCC/DMAP

The copolymer ITA/PLGA-PEG-PLGA/ITA was adjusted by DOPA in the presence of organic solvent N,N-Dimethylformamide (DMF) and activating system consists of Dicyclohexylcarbodiimide with 4-(Dimethylamino)pyridine (DCC/DMAP). Subsequently, butylamine was linked to obtained DOPA-ITA/PLGA-

PEG-PLGA/ITA-DOPA copolymer. The synthesis was proceeded at the room temperature 25 °C. All components was degassed and dissolved in DMF separately.

In the first step, the solution of DCC and DMAP (97.4 µmol, 97.4 µmol, 2.5 mL) was injected to the solution of purified copolymer (81.2 µmol, 4 mL) under the nitrogen atmosphere. The activation lasted for 1 hour at the sample labeled 20170822 and for 2 hours for the sample labeled 20171011 under the stirring (Fig. 26).

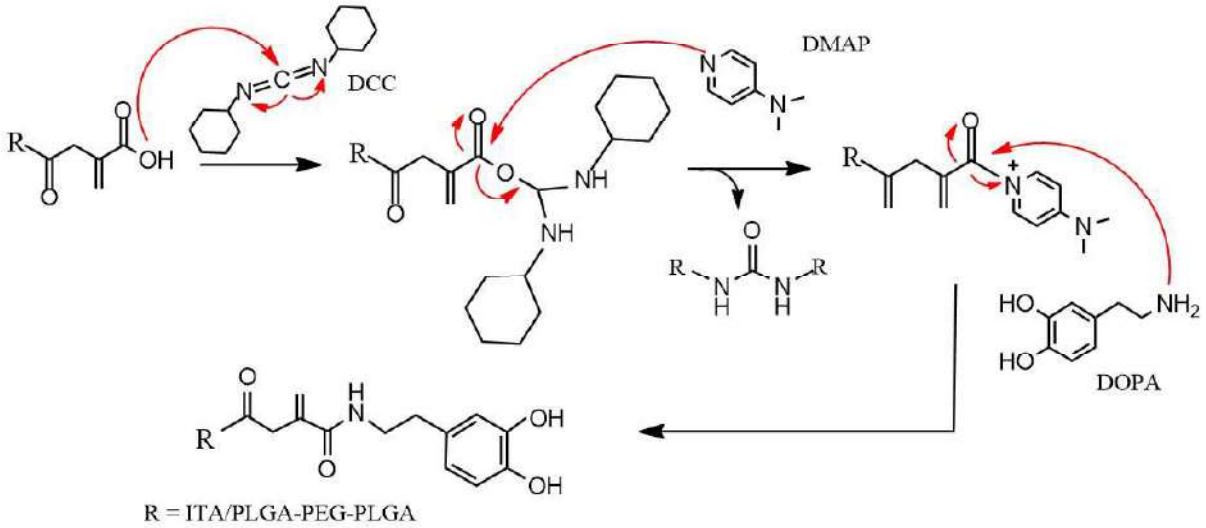

Fig. 26: Reaction mechanism of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA synthesis in the presence of activating system DCC/DMAP.

The second step included the addition of dopamine solution (97.4 µmol, 2.5 mL) and the reaction took 1 hour. After the lapse of reaction time, the reaction was terminated or the synthesis continued by the third step. In the third step the bioactive compound butylamine was linked. Prepared butylamine solution (97.4 µmol, 1 mL) was dropped into the reaction mixture and left to react for 2 hours (Fig. 27). Total volume of DMF was 10 mL.

The final product of synthesis, DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA (ABA/ITA-DOPA) copolymer with linked dopamine or Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine (ABA/ITA-DOPA-ButA) with both dopamine and butylamine, was precipitated into DEE chilled by liquid nitrogen. Precipitated copolymer was dried in vacum oven at 60 °C until the constant weight. Dried product had brown colour. Particular samples are presented in the table 2.

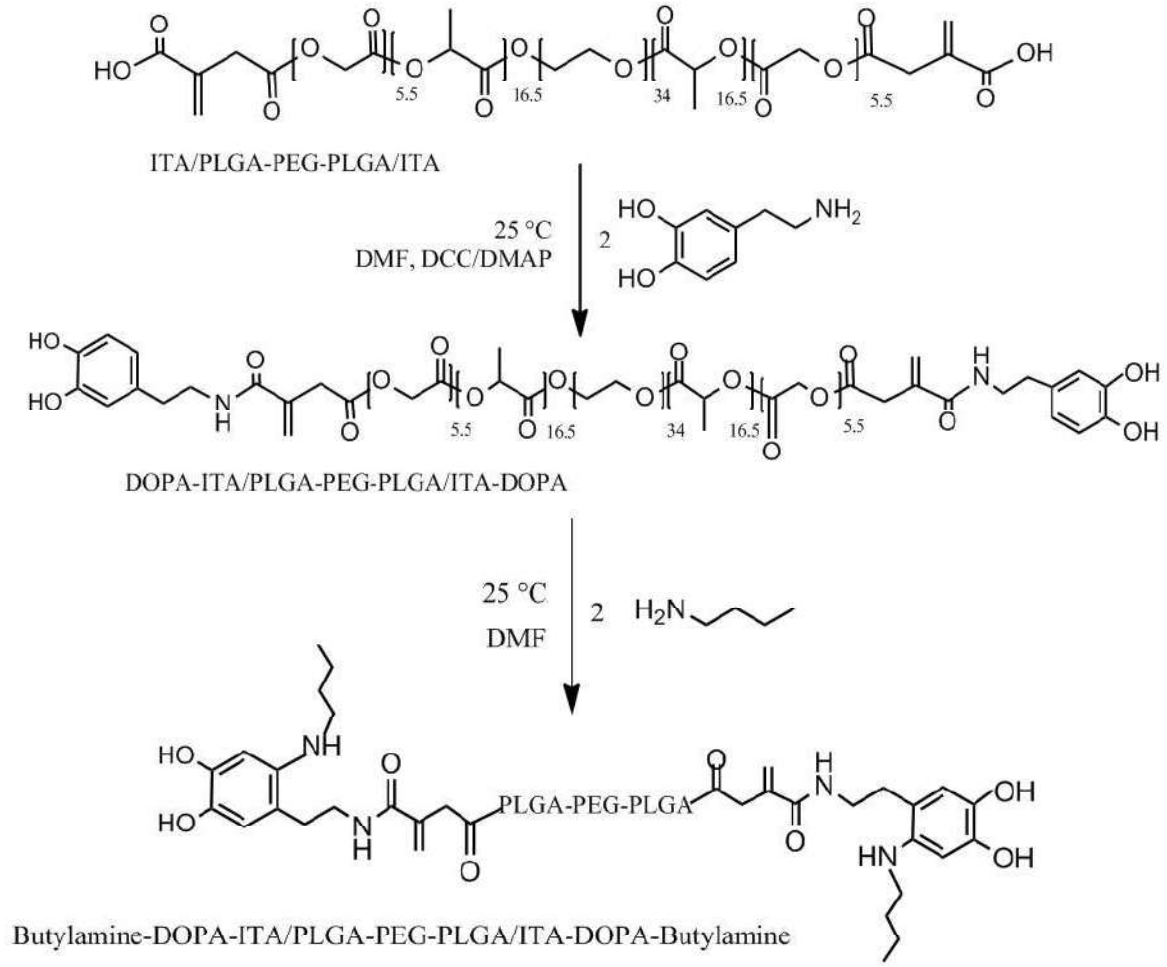

Fig. 27: Reaction scheme of Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine synthesis in the presence of activating system DCC/DMAP.

4.3.2.3 Dopamine and L-lysine Modification in a Bulk

The two-step synthesis of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA copolymer was "one-pot" reaction and proceeded with no solvent in a bulk under the nitrogen atmosphere. The first step was the synthesis of functionalized copolymer ITA/PLGA-PEG-PLGA/ITA (chapter 4.3.1.1) and the following step, the second one, was the modification of copolymer by dopamine. To obtain Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys copolymer, the synthesis proceeded by the third step, linking of the bioactive substance L-lysine.

Dopamine (4.3 mmol) was added to cooled melting of copolymer ITA/PLGA-PEG-PLGA/ITA and degassed carefully. The temperature of reactive mixture was increased to 100 °C and the reaction time was 2 hours under the nitrogen atmosphere. Obtained polymer was purified (chapter 4.3.2.4), dissolved in the solution of L-lysine (4.3 mmol, 50 mL) in the presence of tris-(hydroxymethyl)aminomethane chlorhydrate (TRIS) (5 μmol) and left overnight to react under the stirring at the room temperature with the access of air (Fig. 28). TRIS, a buffer agent, regulated pH to the value 8.5, the value of the highest dopamine reactivity. Modified copolymer was dark brown coloured. Particular samples are presented in the table 2.

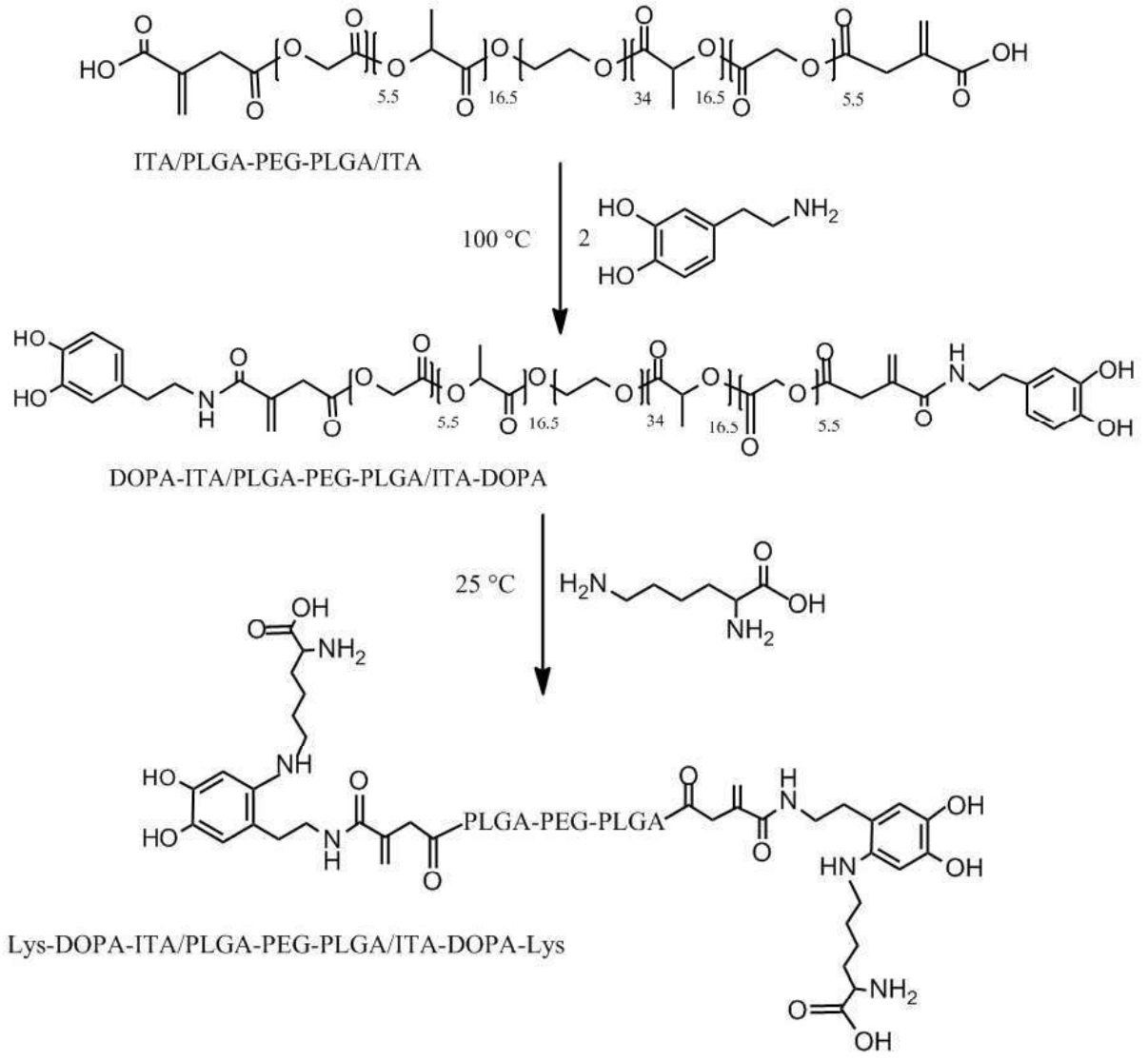

Fig. 28: Reaction scheme of Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys synthesis.

4.3.2.4 The Purification of Samples

A cycle of purification included the dissolution of prepared copolymer in ultrapure water to purge of unreacted monomers, the precipitation of copolymer at the temperature 80 °C to separate it from impure water and the decantation of precipitated copolymer to isolate it. During the purification process, limited concentration of copolymer solution was 10 %. If the concentration was lower, the copolymer could not be precipitated. The purification process involved three cycles which were repeated and purified products were freeze-dried by means of lyophilisation. The appearance of final products is presented in the figure 29.

Table 2: Summary of synthesized samples.

Number	Sample	Synthesis	Activating system
20170306	ABA/ITA	Aqueous solution	-
20170306	ABA/ITA-DOPA		
20170720	ABA/ITA-DOPA		EDC/NHS
20170329	ABA/ITA	In a bulk	-
20170831	ABA/ITA-DOPA	Organic solution (DMF)	DCC/DMAP
20170822	ABA/ITA-DOPA-ButA		
20171010	ABA/ITA		
20171011	ABA/ITA-DOPA-ButA		
20180226	ABA/ITA	In a bulk	-
20180226	ABA/ITA-DOPA		
20180305	ABA/ITA		
20180305	ABA/ITA-DOPA		
20180305	ABA/ITA-DOPA-Lys		

Fig. 29: Purified and dried samples (white ABA, yellow ABA/ITA, brown ABA/ITA-DOPA and ABA/ITA-DOPA-ButA, dark ABA/ITA-DOPA-Lys).

4.4 Characterization Methods

Prepared samples was characterized precisely by means of Nuclear magnetic rezonance spectroscopy (^{1}H-NMR), Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy (ATR-FTIR) and Dynamic Rheological Analysis (DRA) analyses.

4.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

Polymer characterization and its molecular weight were confirmed using ^{1}H-NMR spectroscopy. Spectra were recorded on 700 MHz Bruker AVANCE III HD instrument using 128 scans. Samples were in form of solution in deuterated chloroform ($CDCl_3$) at the temperature 25 °C. Chemical shifts were reported in ppm relative to tetramethylsilane (TMS). ^{1}H-NMR spectra were proceeded using ACD/1D NMR Processor. Measurement was provided by RNDr. O. Humpa from Masaryk University in Brno.

4.4.2 Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy (ATR-FTIR)

IR spectra of samples were confirmed by attenuated total reflectance Fourier transformed infrared spectrometer (ATR-FTIR) Bruker Tensor 27 with diamond ATR crystal in a range between 4000-600 cm^{-1}, 32 scans at 4 cm^{-1} resolution. Spectra were measured at room temperature and evaluated by OPUS software.

4.4.3 Dynamic Rheological Analysis (DRA)

Rheological properties and the sol-gel transition of the copolymer solutions were detected in the linear viscoelastic region. The temperature dependence measurement was proceeded on a rheometer TA Instruments AR-2 with cone-plate sensor geometry with diameter 40 mm and 2° angle. The experiments were carried out under the constant frequency of 1 rad s^{-1} and 1 % strain. The temperature ramp was set in the range between 20 - 60 °C and the rate was 0.5 °C min^{-1}.

5 RESULT AND DISCUSSION

Functionalized copolymer ITA/PLGA-PEG-PLGA/ITA was prepared in order to obtain reactive double bonds and carboxylic groups appropriate for the modification. Synthesis named ring-opening polymerization was proceeded in a bulk. The next step included the attachment of dopamine, bioactive, adhesive and stabilizing agent. The attachment was carried out in aqueous or organic solution and in a bulk with different usage of activating systems either EDC/NHS or DCC/DMAP. The following step of modifying synthesis was linking of the butylamine and L-lysine as a bioactive compounds with relatively simple structure. The simplification offers the expectation of protein reaction mechanism.

5.1 Synthesis of Funcionalized Copolymers

Firstly, functionalized copolymer ITA/PLGA-PEG-PLGA/ITA was synthesized in order to obtain reactive double bonds and carboxylic groups suitable for consequent modification. The copolymerization was proceeded via ring-opening mechanism in a bulk.

5.1.1 Synthesis of ITA/PLGA-PEG-PLGA/ITA

The ring-opening copolymerization of D,L-lactide, glycolide and macroinitiator poly(ethylene glycol) with using the catalyst Sn-octoate led to the preparation of PLGA-PEG-PLGA triblock copolymer. Following reaction with ITA was functionalization of original copolymer and it formed ITA/PLGA-PEG-PLGA/ITA triblock copolymer (Fig. 30). All purified samples were characterized by ^{1}H NMR, ATR FTIR and DRA analysis. The next chapters introduce only one spectrum because of the spectra of all samples ITA/PLGA-PEG-PLGA/ITA (ABA/ITA) were the same.

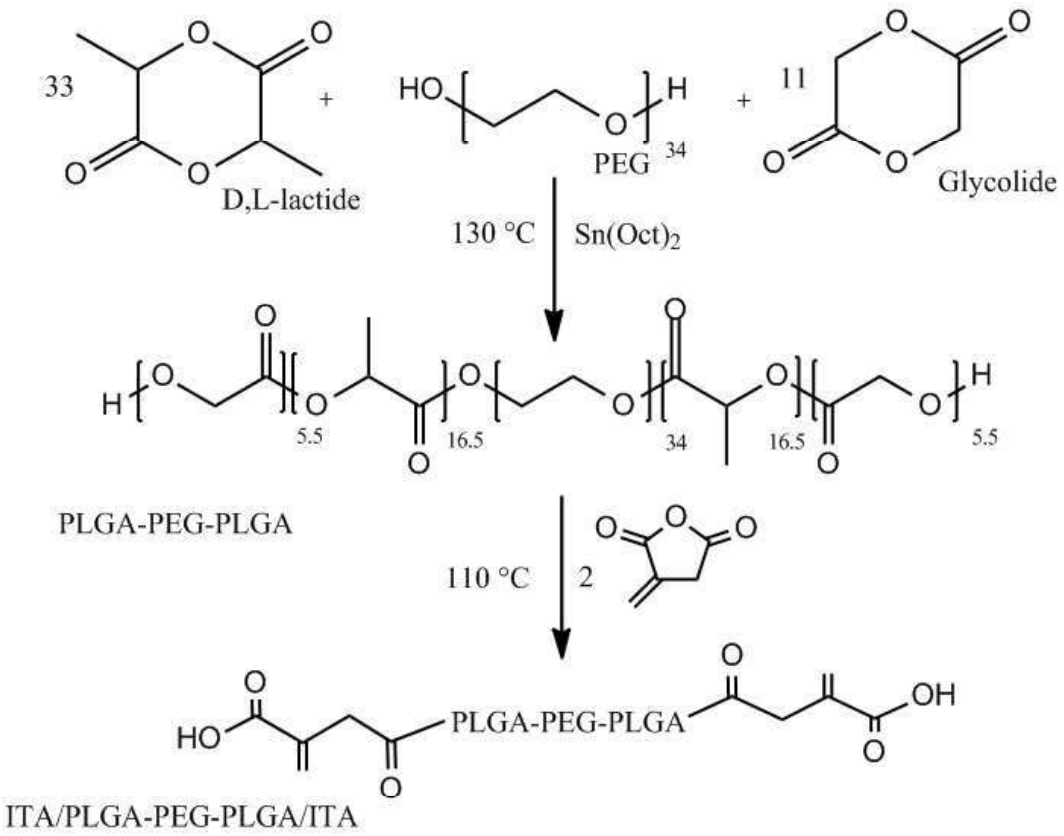

Fig. 30: Synthesis scheme of ITA/PLGA-PEG-PLGA/ITA triblock copolymers.

5.1.1.1 Characterization by Proton Nuclear Magnetic Resonance Spectroscopy

Prepared samples of synthesized copolymers were characterized by the means of ^{1}H NMR spectroscopy and the outcomes of the measurements were NMR spectra. NMR spectrum of functionalized ITA/PLGA-PEG-PLGA/ITA copolymer showed several signals (Fig. 31).

Characteristic signals of lactic acid protons (O-(C$\underline{H}_3$)C$\underline{H}$O) were found in the range between 5.14 - 5.29 ppm (multiplet, 1H) (peak A) and 1.46 - 1.63 ppm (multiplet, 3H) (peak E). Protons of glycolide acid (OC$\underline{H}_2$O) had characteristic signal in range between 4.61 - 4.92 ppm (multiplet, 2H) (peak B) and signals belonged to protons of poly(ethylene glycol) (OC$\underline{H}_2$C$\underline{H}_2$O) was in the range between 3.55 - 3.74 ppm (multiplet, 3H) (peak D) and (-O-CH$_2$C$\underline{H}_2$-O-) between 4.23 - 4.33 ppm (multiplet, 2H) (peak C).

Characteristic signal of itaconic acid backbone (OC(C$\underline{H}_2$)CC$\underline{H}_2$COOH) were found in the range between 3.43 - 3.55 ppm (singlet, 1H) (peak H) and protons of itaconic acid double bonds had signals in the range between 5.78 - 5.89 ppm (singlet, 1H) (peak G) and 6.37 - 6.47 ppm (singlet, 1H) (peak F) [2]. Peak at 7.25 ppm was characteristic signal for solvent $CDCl_3$.

Real molecular weight of prepared copolymers was determined from integrals of lactic acid and glycolic acid signals labeled A and B. The amount of end-capped ITA was confirmed from integrals of its characteristic signal F.

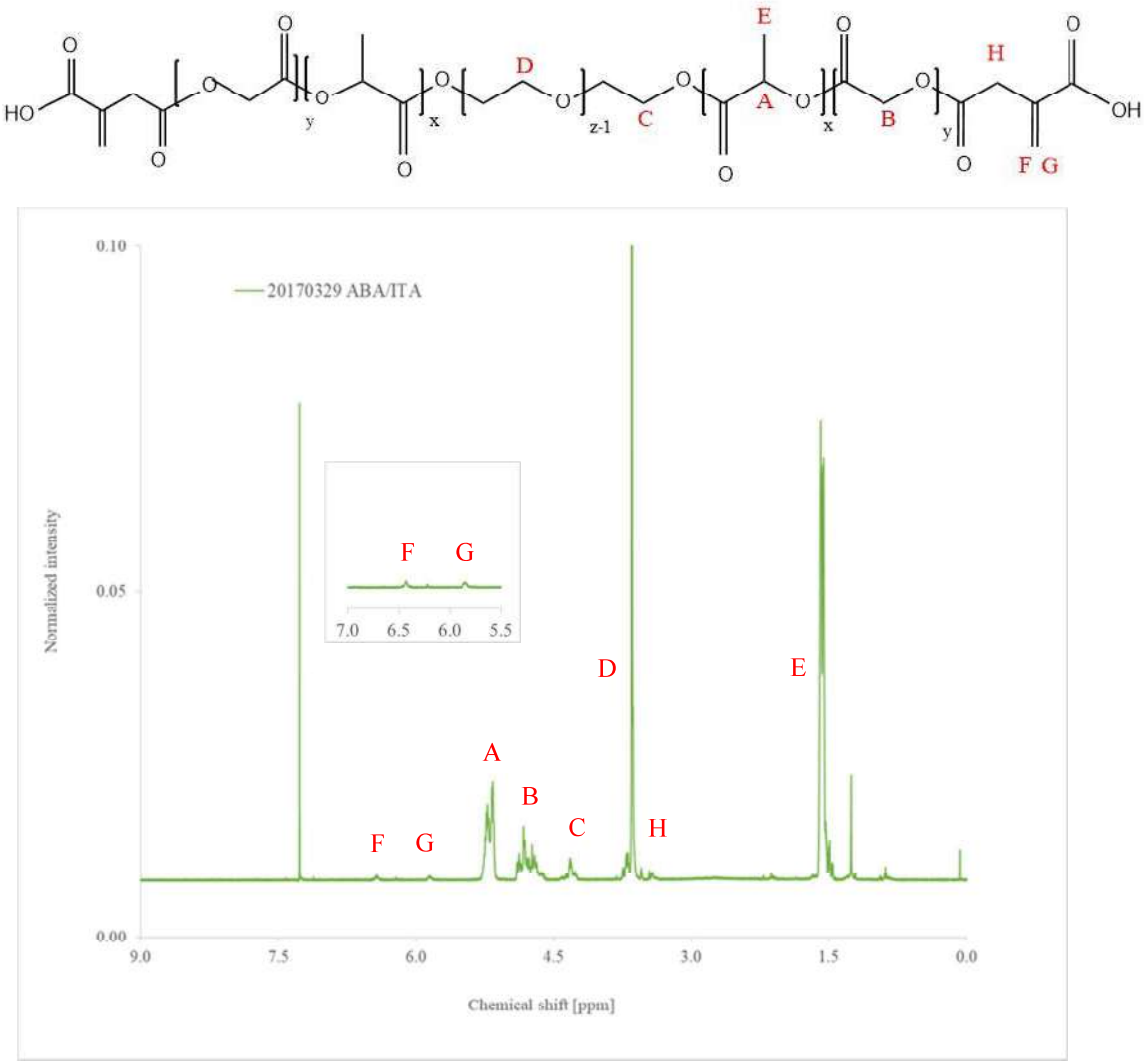

Fig. 31: ^{1}H NMR spectrum of ITA/PLGA-PEG-PLGA/ITA triblock copolymer.

Altogether, 5 samples of ABA/ITA was synthesized. Their real molecular weights and PLGA/PEG and LA/GA ratio are compared to the theoretical ones in the table 3. Molecular weight calculated from

NMR spectra was consistent with theoretical molecular weight of copolymers except the sample labeled 20170306 ABA/ITA. The deviation was caused by inaccurate dosing of PEG probably. The amount of bonded ITA ranged between 51.5 - 79.4 mol %. Higher amount of bonded ITA was the consequence of more intensive stirring of viscous reactive mixture.

Table 3: Summary of synthesized samples and their characteristics.

Sample	ITA	M_n [g mol^{-1}]		PLGA/PEG [wt/wt]		LA/GA [mol/mol]	
ABA/ITA	[mol %]	Theor	NMR	Theor	NMR	Theor	NMR
20170329	76.2		4657		2.1		2.9
20170306	51.5		5503		2.6		3.1
20171010	57.4	4500	4598	2.0	2.0	3.0	2.9
20180226	71.1		4587		2.0		3.0
20180305	79.4		4699		2.1		2.7

* Theor – theoretical, NMR – calculated from NMR spectrum

5.1.1.2 Characterization by Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy

The FTIR spectroscopy confirmed the structural character of prepared ITA/PLGA-PEG-PLGA/ITA copolymers and completed the identification of the material. Obtained infrared spectrum involved several peaks characterizing particular functional groups (Fig. 32).

Ester groups appeared at around 1100 cm^{-1} and carbonyl groups were represented by thin peak at 1780 cm^{-1}. Characteristic peaks of alifatic alkyl groups were observed at 2850 - 2990 cm^{-1} and broad peak at around 3450 cm^{-1} showed carboxylic functional groups which end-capped the copolymer.

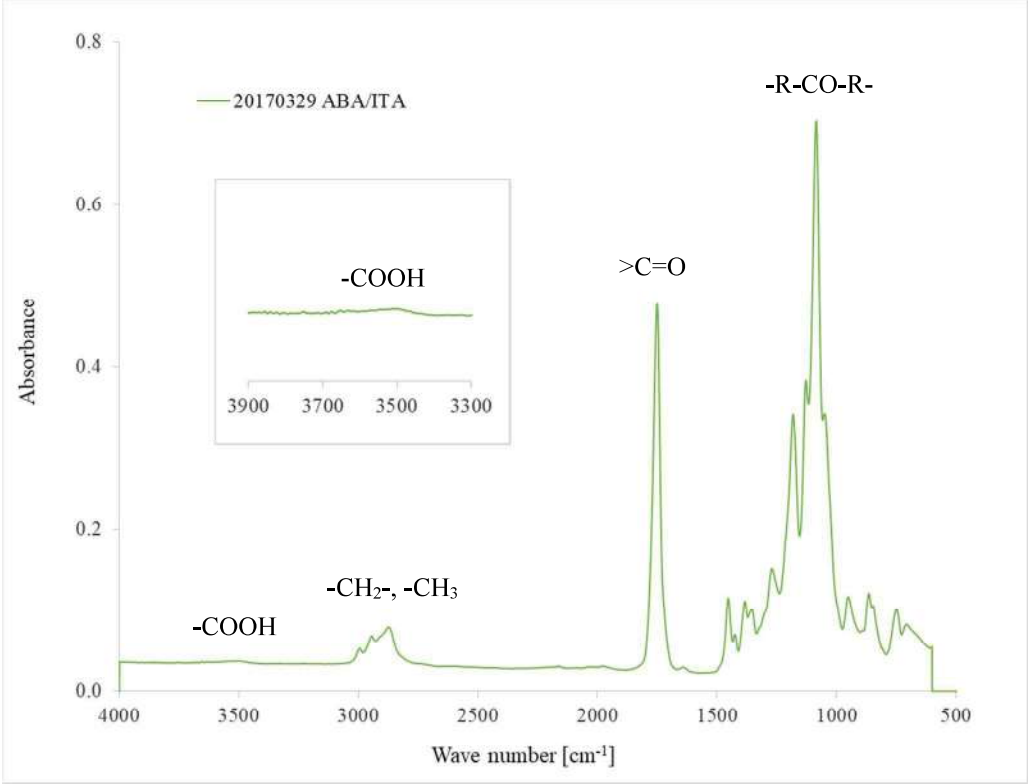

Fig. 32: FTIR spectrum of ITA/PLGA-PEG-PLGA/ITA triblock copolymer.

5.1.1.3 Characterization by Dynamic Rheological Analysis

Dynamic rheological analysis is a method used to study materials with viscoelastic behaviour, especially polymers. The result of measurement was temperature dependence of storage and loss

modulus. Storage modulus G´presented the elastic character and the loss modulus G´´ presented the viscous character of material. In the figure 33, there were two crosses of storage modulus curve and loss modulus curve.

The first cross at 30 °C was considered to be the critical point of gelation temperature, the transition from viscous liquid to solid gel of ITA functionalized copolymer PLGA-PEG-PLGA (20 % aqueous solution). While the physical gel was forming, the temperature and the both modulus were increased to the maximum of storage modulus at 71.6 Pa, 35.0 °C and to the maximum of loss modulus at 58.8 Pa, 35.7 °C. The maximum of storage modulus defines the stiffness of formed gel. The second cross at 36.7 °C was regarded as the end of the gelation process.

The functionalization by ITA moved the gel point to the lower temperature in comparison to unmodified copolym**er** PLGA-PEG-PLGA [20].

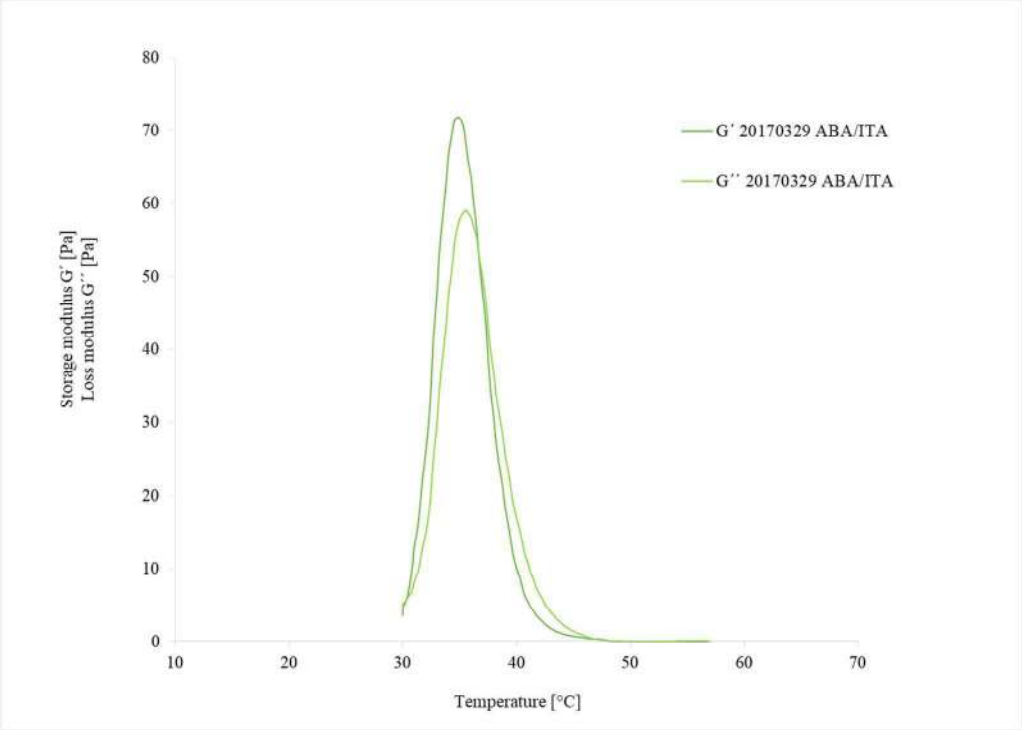

Fig. 33: Rheological properties of PLGA-PEG-PLGA and ITA/PLGA-PEG-PLGA/ITA triblock copolymers.

5.2 Synthesis of Modified Copolymers

The second step of synthesis included the attachment of dopamine like bioactive, adhesive and stabilizing agent. The attachment was proceeded in aqueous or organic solution and in a bulk with or without activating systems (EDC/NHS, DCC/DMAP). The third step of modifying synthesis was linking of the butylamine and L-lysine as a bioactive compounds with relatively simple structure that replaces the complex structure of proteins.

5.2.1 Dopamine Modification in Aqueous Solution

The high water content in the living body ensures the aqueous environment natural for whole scale of metabolic ways and biological reactions. First experiments was aimed at synthesis proceeded in environment natural to the organism without using organic solvents and chemical substances.

5.2.1.1 Without the Usage of Activating System

The synthesis proceeded in the aqueous solution of ITA functionalized copolymer and dopamine under the mild conditions. The reaction scheme is presented in the figure 34.

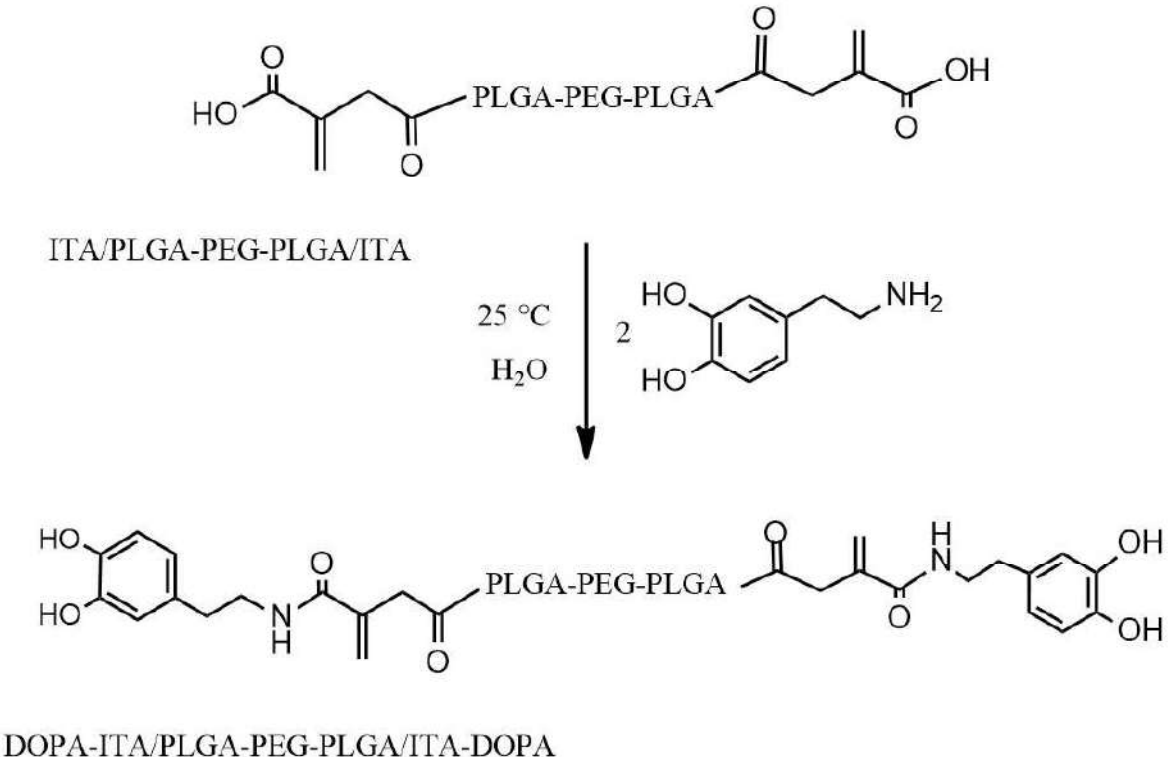

Fig. 34: Synthesis scheme of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

5.2.1.1.1 Characterization by Proton Nuclear Magnetic Resonance Spectroscopy

Characteristics of dopamine modified copolymers were obtained by the means of ^{1}H NMR spectroscopy and NMR spectrum of modified copolymer (Fig. 35) is described below.

Typical signals of lactic acid protons (A, E), glycolide acid protons (B), poly(ethylene glycol) protons (D, C) and itaconic acid protons (F, G, H) appeared.Characteristic signals of lactic acid protons (O-(C$\underline{H_3}$)C$\underline{H}$O) were found in the range between 5.14 - 5.29 ppm (multiplet, 1H) (peak A) and 1.46 - 1.63 ppm (multiplet, 3H) (peak E). Protons of glycolide acid (OC$\underline{H_2}$O) had characteristic signal in range between 4.61 - 4.92 ppm (multiplet, 2H) (peak B) and signal belonged to protons of poly(ethylene glycol) (OC$\underline{H_2}$C$\underline{H_2}$O) was in the range between 3.55 - 3.74 ppm (multiplet, 3H) (peak D) and (-O-CH$_2$C$\underline{H_2}$-O-) between 4.23 - 4.33 ppm (multiplet, 2H) (peak C).

Characteristic signal of itaconic acid backbone (OC(C$\underline{H_2}$)CC$\underline{H_2}$COOH) were found in range between 3.43 - 3.55 ppm (singlet, 1H) (peak H) and protons of itaconic acid double bonds had signals in range between 5.78 - 5.89 ppm (singlet, 1H) (peak G) and 6.37 - 6.47 ppm (singlet, 1H) (peak F) [2].

In according to ^{1}H NMR prediction, dopamine´s characteristic signals of protons ((OH)$_2\underline{Ph}$CH$_2$CH$_2$NH$_2$) were expected in the range between 6.69 - 6.80 ppm (multiplet, 3H) (peak I) and ((OH)$_2$PhC$\underline{H_2}$C$\underline{H_2}$NH$_2$) protons´ signals ranged from 2.79 to 3.07 ppm (triplets, 4H) (peaks J). In the NMR spectrum of sample 20170306 ABA/ITA-DOPA was obvious small peaks J of dopamine protons, but it was assigned to formed polydopamine coating considering the result of infrared spectra that did not showed any peptide bonds (Fig. 36). There was also characteristic signal of solvent $CDCl_3$ with typical peak at 7.25 ppm.

Calculated molecular weight of prepared copolymers was determined from integrals of lactic acid and glycolic acid signals labelled A and B. The amount of end-capped ITA was confirmed from integrals of its characteristic signal F.

Real molecular weights and PLGA/PEG and LA/GA ratio are compared in the table 4. NMR spectrum was used to calculate molecular weight of prepared copolymers. Real and theoretical values was not in good agreement. The deviation caused by inaccurate dosing of PEG was displayed in the spectra of both samples.

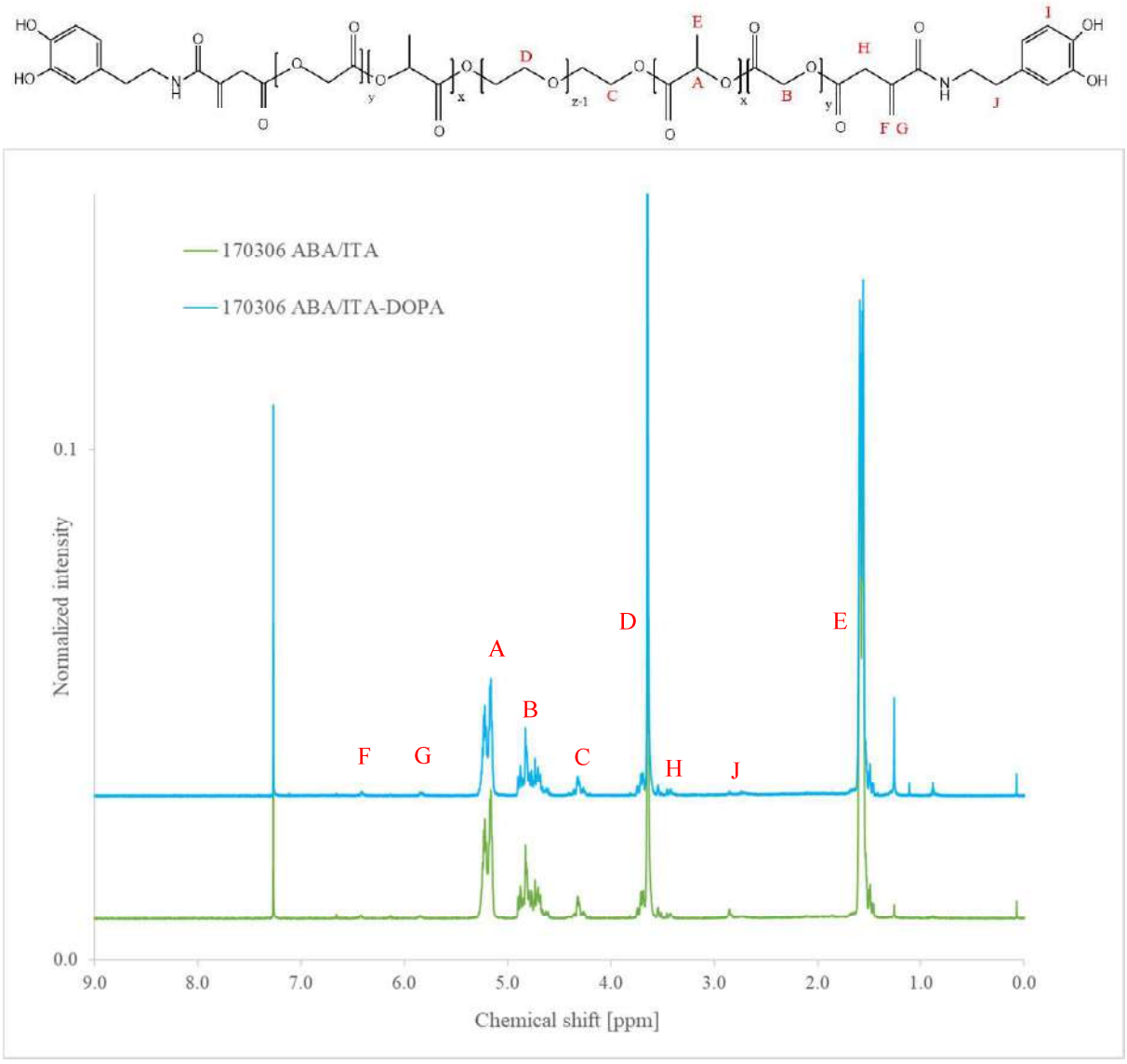

Fig. 35: ^{1}H NMR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

Table 4: Summary of synthesized samples and their characteristics.

Sample	ITA	M_n [g mol^{-1}]		PLGA/PEG [wt/wt]		LA/GA [mol/mol]	
	[mol %]	Theor	NMR	Theor	NMR	Theor	NMR
20170306 ABA/ITA	51.5	4500	5503	2.0	2.67	3.0	3.05
20170306 ABA/ITA-DOPA	49.7		5498		2.67		3.06

5.2.1.1.2 Characterization by Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy

Infrared spectrum measured by the means of ATR method supported the result of NMR spectroscopy. Except already identified functional groups, ester groups at around 1100 cm^{-1}, carbonyl groups presented by thin peak at 1780 cm^{-1}, characteristic peaks of alifatic alkyl groups observed at 2850 - 2990 cm^{-1} and broad peak of carboxylic groups at around 3450 cm^{-1}, we did not observe any other new peak, as expected (Fig. 36).

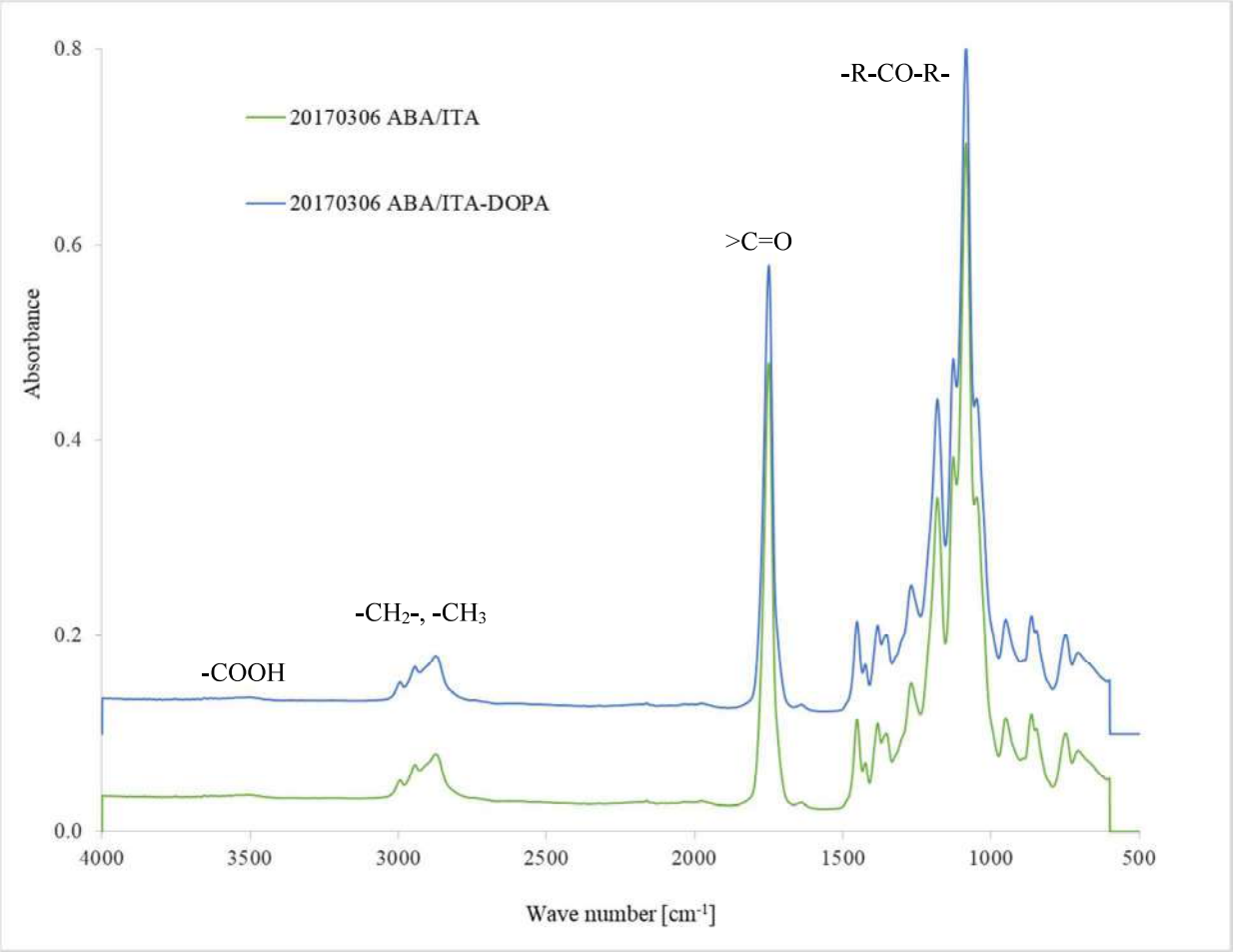

Fig. 36: FTIR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

5.2.1.1.3 Characterization by Dynamic Rheological Analysis

Dynamic rheological analysis was used to measure the viscoelastic behaviour of dopamine modified copolymers. The temperature dependence of storage and loss modulus was the outcome of the measurement. Storage modulus G´ represented the elastic character and the loss modulus G´´ represented the viscous character of the material. There were observed two crosses of storage modulus and loss modulus curve.

In the figure 37, the first cross of G´and G´´ of sample 20170306 ABA/ITA DOPA was at the value of temperature 30 °C. The first cross was critical point of the sol-gel transition of DOPA modified copolymer ITA/PLGA-PEG-PLGA/ITA (20 % solution). While the physical gel was forming, the temperature and the both modulus increased. The maximum of storage modulus settled at 71.6 Pa, 35.0 °C and the maximum of loss modulus settled at 58.8 Pa, 35.7 °C. The second cross at 36.7 °C was considered to be the end of the process of gelation.

Dynamic rheological analysis confirmed the results of ^{1}H NMR spectroscopy and FTIR spectroscopy. The rheological properties, gelation behaviour or critical gel temperature were not changed in comparison to ITA/PLGA-PEG-PLGA/ITA copolymer (Fig. 37).

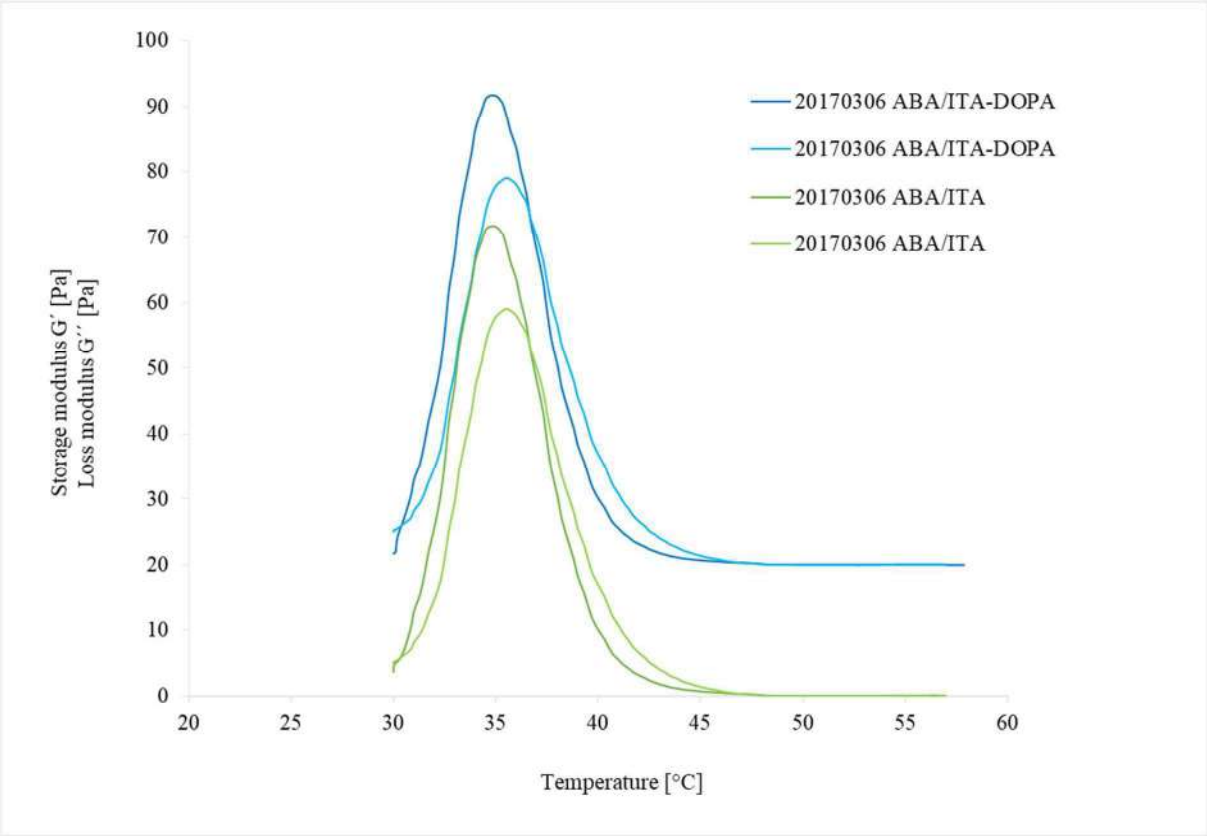

Fig. 37: Rheological properties of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

5.2.1.2 In the Presence of Activating System EDC/NHS

The synthesis in the aqueous solution of ITA functionalized copolymer and dopamine proceeded under the mild conditions in the presence of activating system EDC/NHS. The reaction scheme is presented in the figure 38 and the reaction mechanism is explained in the figure 23, chapter 4.3.2.1.2. The experiment was unsuccessful because of formation of polydopamine that proved the change of sample´s colour from white to black and the chemical bonding of dopamine was contradicted. Furthermore, the character of sample changed from viscous liquid to solid as shown in the figure 39 and that is the reason why the sample was not analysed.

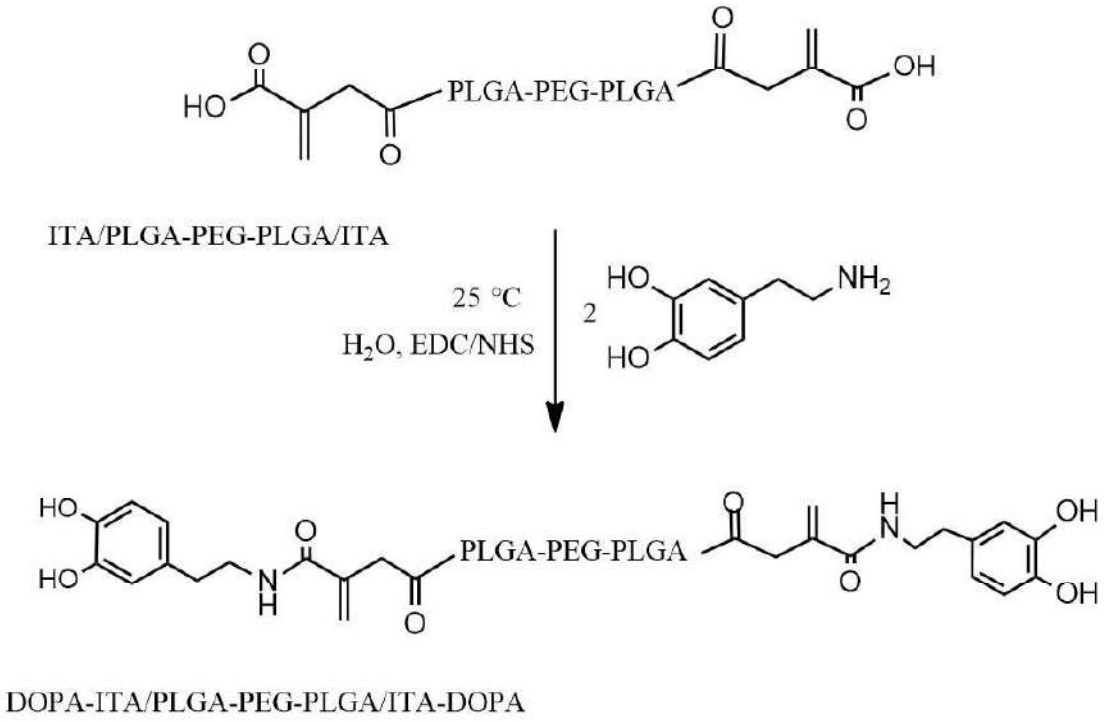

Fig. 38: Synthesis scheme of DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

Fig. 39: Sample prepared by synthesis in aqueous solution with activating system EDC/NHS.

5.2.2 Dopamine and Butylamine Modification in Organic Solution in the Presence of Activating System DCC/DMAP

After unsuccessful experiments in aqueous solution, the water was replaced by solvent DMF (chosen in dissolubility test, table 1 in chapter 4.3.2.2) and the activating system DCC/DMAP was used. The reaction scheme is showed below (Fig. 40) and the reaction mechanism of carboxylic groups activation is described in chapter 3.3.2.2.1.

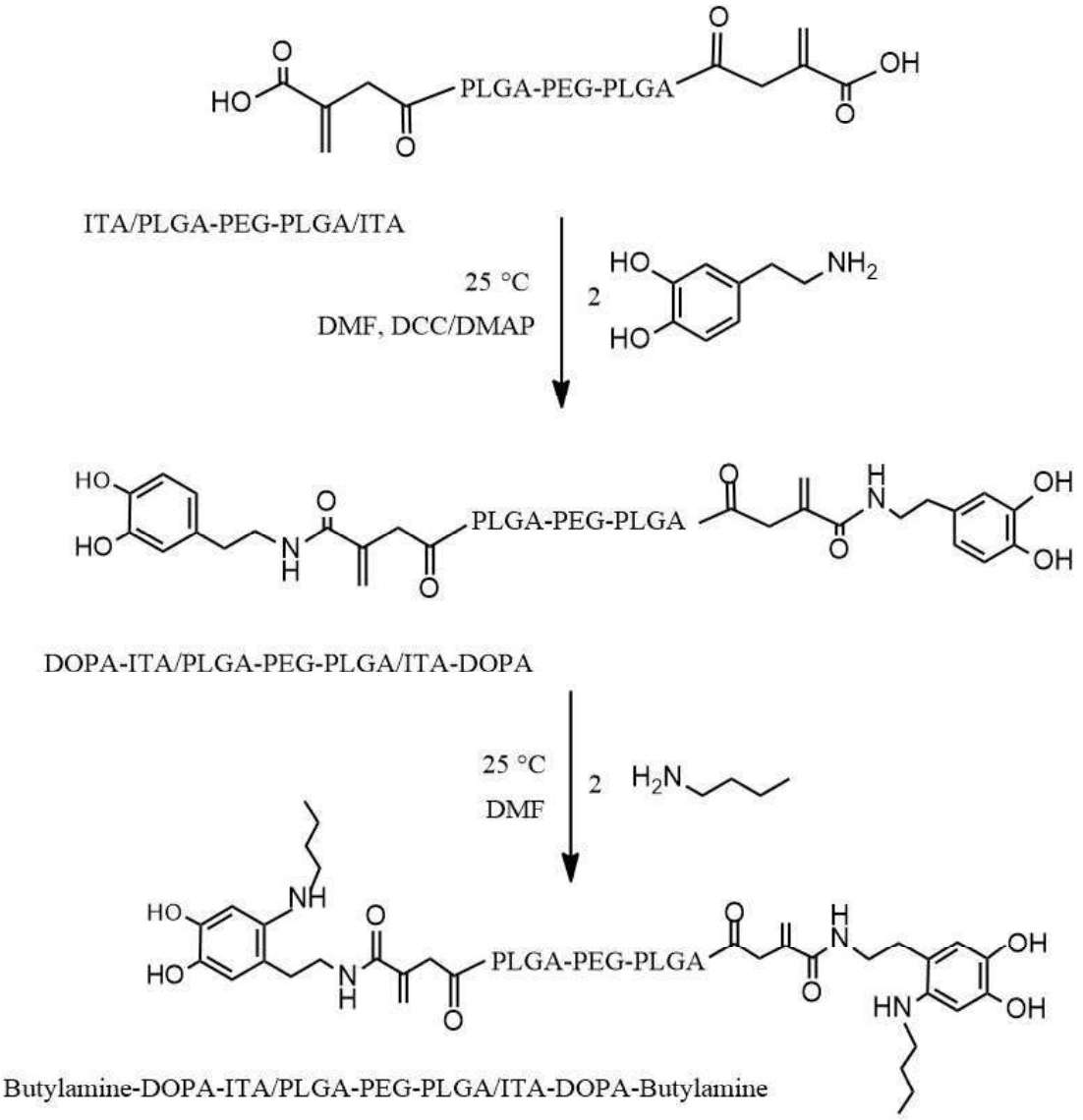

Fig. 40: Synthesis scheme of Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine triblock copolymers.

5.2.2.1 Characterization by Proton Nuclear Magnetic Resonance Spectroscopy

ITA/PLGA-PEG-PLGA/ITA copolymers modified by dopamine and butylamine were analysed by the means of ^{1}H NMR spectroscopy and obtained NMR spectrum (Fig. 41) is discussed below. The allocation of signals characterizing protons of lactic acid (A, E), glycolide acid (B), poly(ethylene glycol) (D, C) and itaconic acid (F, G, H) appeared. Characteristic signals of lactic acid protons (O-(C$\underline{H}_3$)C$\underline{H}$O) were found in the range between 5.14 - 5.29 ppm (multiplet, 1H) (peak A) and 1.46 - 1.63 ppm (multiplet, 3H) (peak E). Protons of glycolide acid (OC$\underline{H}_2$O) had characteristic signal

in range between 4.61 - 4.92 ppm (multiplet, 2H) (peak B) and signal belonged to protons of poly(ethylene glycol) ($OC\underline{H_2}C\underline{H_2}O$) was in the range between 3.55 - 3.74 ppm (multiplet, 3H) (peak D) and ($-O-CH_2C\underline{H_2}-O-$) between 4.23 - 4.33 ppm (multiplet, 2H) (peak C).

Characteristic signal of itaconic acid backbone ($OC(C\underline{H_2})CC\underline{H_2}COOH$) were found in range between 3.43 - 3.55 ppm (singlet, 1H) (peak H) and protons of itaconic acid double bonds had signals in range between 5.78 - 5.89 ppm (singlet, 1H) (peak G) and 6.37 - 6.47 ppm (singlet, 1H) (peak F) [2].

Signals characteristic for dopamine protons ($(OH)_2\underline{Ph}CH_2CH_2NH_2$) were found in the range between 6.69 - 6.80 ppm (multiplet, 3H) (peak I) and ($(OH)_2PhC\underline{H_2}C\underline{H_2}NH_2$) protons´ signals ranged from 2.79 to 3.07 ppm (triplets, 4H) (peak J). Protons of butylamine ($C\underline{H_3}CH_2CH_2CH_2NH_2$) were found in the range between 1.20 - 1.22 ppm (triplet, 3H) (peak L). Typical signals for another protons were covered by more intensive multiplets of protons belonged to PLGA-PEG-PLGA copolymer.

In the spectrum of modified copolymers´samples could be seen peak at 8.1 ppm (singlet, 1H) typical for solvent DMF, at 7.25 ppm was peak characteristic for solvent $CDCl_3$ (singlet, 1D). The sample 20170831 contained the compound of activating system DMAP with characteristic peak at 3.2 ppm (singlet, 6H) and peak at 8.3 ppm (doublet, 2H). Samples 20170822 and 20171011 contained solvent DEE with its typical peak at 3.4 ppm (kvadruplet, 4H). Signals belonged to solvents DMF and DEE showed that the samples was not dried until the constant weight.

The amount of dopamine was determined from integrals of obtained signals with marks J and integrated signal marked L offered the determination of butylamine amount. The amount of bonded dopamine ranged between 7.7 - 18.6 mol % and in the proportion to bonded ITA was the range from 10.1 to 32.4 mol %. The amount of bonded butylamine ranged between 4.5 - 7.8 mol %, in the proportion to bonded ITA ranged from 5.4 to 13.5 mol % and in the proportion to bonded DOPA was the range from 48.0 to 41.8 mol %. The particular values are assigned in the tables 5 and 6.

Table 5: The comparison of real and theoretical values of observed characteristics.

Sample	M_n [g mol^{-1}]		PLGA/PEG [wt/wt]		LA/GA [mol/mol]	
	Theor	NMR	Theor	NMR	Theor	NMR
20170329 ABA/ITA		4657		2.1		2.9
20170831 ABA/ITA-DOPA		4560		2.0		3.1
20170822 ABA/ITA-DOPA-ButA	4500	4646	2.0	2.1	3.0	2.9
20171010 ABA/ITA		4598		2.0		2.9
20171011 ABA/ITA-DOPA		4603		2.0		2.9

Table 6: Summary of dopamine and butylamine bonded amount.

Sample	ITA [mol %]	DOPA [mol %]	DOPA/ITA [mol %]	ButA [mol %]	ButA/ITA [mol %]	ButA/DOPA [mol %]
20170831 ABA/ITA-DOPA	76.2	7.7	10.1	-	-	-
20170822 ABA/ITA-DOPA-ButA		8.5	11.2	4.5	5.4	48.0
20171011 ABA/ITA-DOPA	57.4	18.6	32.4	7.8	13.5	41.8

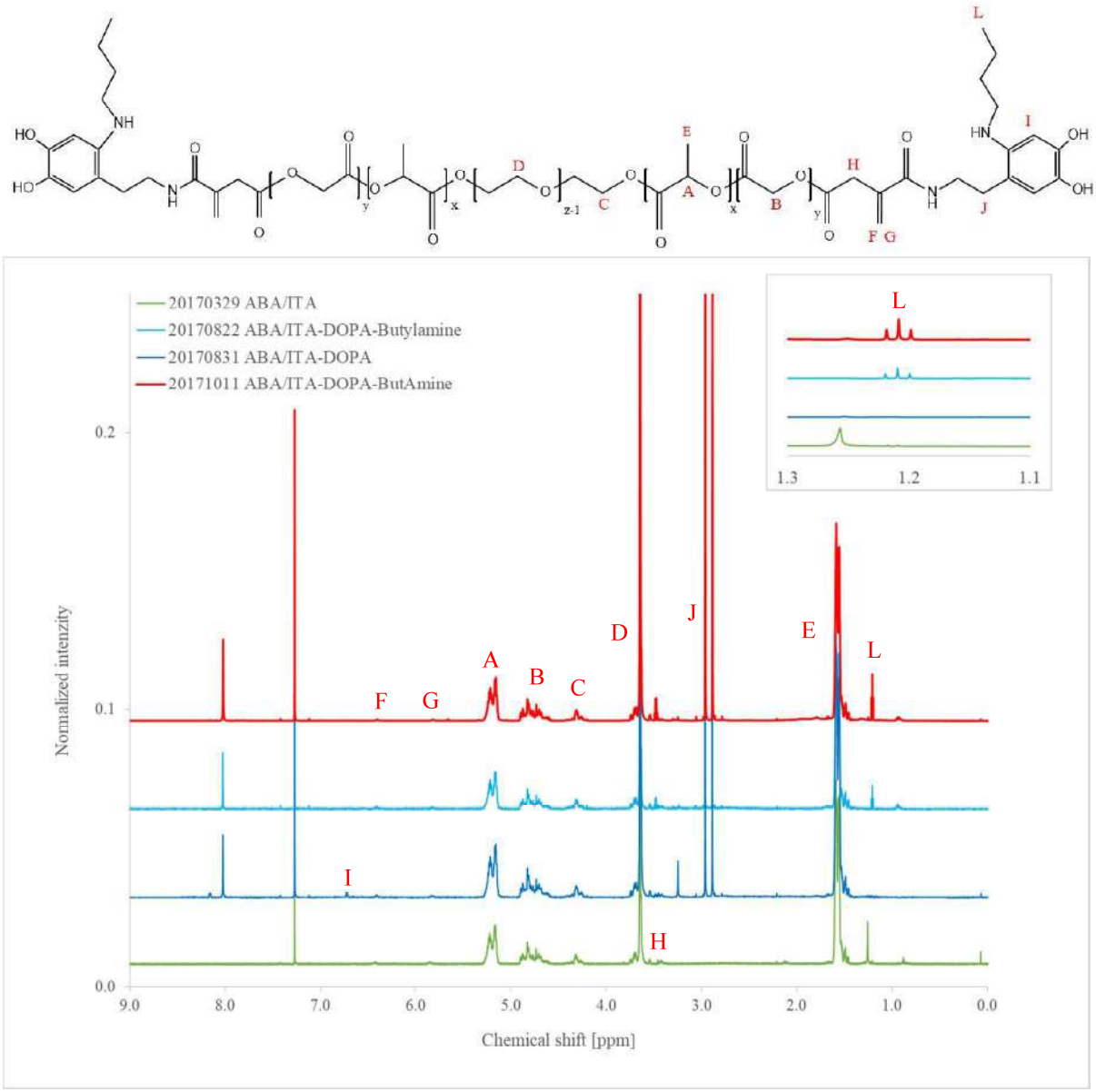

Fig. 41: ^{1}H NMR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA and Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine triblock copolymers.

5.2.2.2 Characterization by Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy

Infrared spectrum of synthesized copolymers confirmed the structural modification of observed polymeric samples. The appearance of peaks chracteristic for ester groups at around 1100 cm^{-1}, carbonyl groups with thin peak at 1780 cm^{-1}, alifatic alkyl groups observed at 2850 - 2990 cm^{-1} affirmed the same structure of basic copolymer ITA/PLGA-PEG-PLGA/ITA when compared to the figure 32.

The change was observed in the presense of peak at 1690 cm^{-1} typical for peptide bonds and it proved the formation of covalent bonds between dopamine and itaconic acid attached to polymer (Fig. 42).

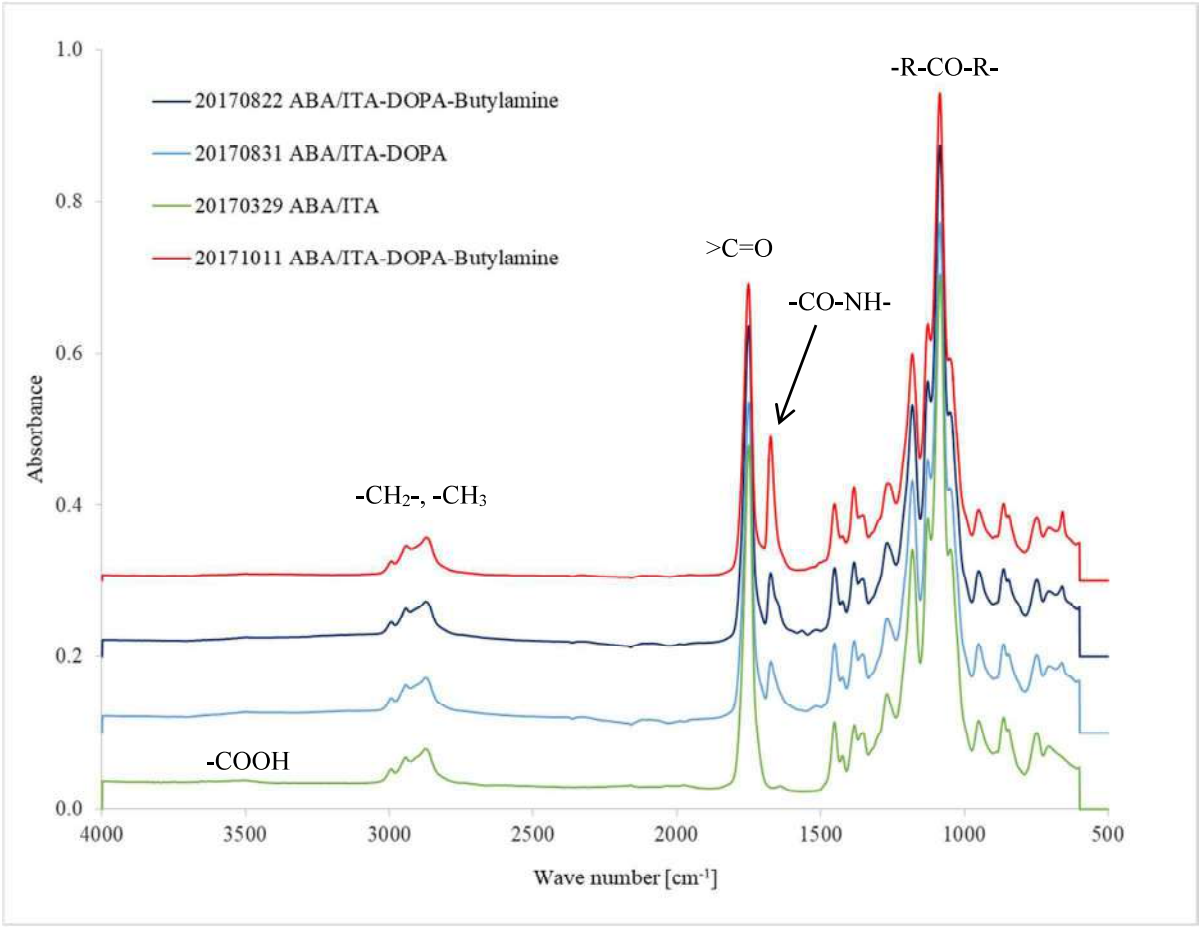

Fig. 42: FTIR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA and Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine triblock copolymers.

5.2.2.3 Characterization by Dynamic Rheological Analysis

The gelation behaviour was observed by the means of Dynamic Rheological analysis and the results of measurement are presented in the figures 43 and 44. There were showed the temperature dependence of storage and loss modulus. Storage modulus G´presented the elastic character and the loss modulus G´´ presented the viscous character of viscoelastic material.

Solution (20 % in ultrapure water) of sample 20170831 ABA/ITA-DOPA showed the first cross, sol-gel transition, at the temperature 32.8 °C. While the physical gel was forming, the temperature and the both modulus were increased to the maximum of storage modulus at 27.9 Pa, 37.3 °C and to the maximum of loss modulus at 17.4 Pa, 37.8 °C. The second cross at 38.3 °C was regarded as the end of the gelation process.

Sample 20170822 ABA/ITA-DOPA-Butylamine (20 % solution in ultrapure water) showed the sol-gel transition at the temperature 31.8 °C. The maximum of storage modulus was at 28.4 Pa, 38.8 °C and the maximum of loss modulus was at 18.9 Pa, 36.2 °C. The second cross at 36.8 °C was observed and showed the end of the gelation process.

Sample labeled 20171011 ABA/ITA-DOPA-Butylamine (20 % solution in ultrapure water) presented the sol-gel transition at the temperature 36.2 °C. The maximum of storage modulus was at 49.9 Pa, 40.0 °C and the maximum of loss modulus was at 49.7 Pa, 40.5 °C. The second cross at 40.5 °C was the end of the forming gel.

In all cases the modifications influenced the temperatures of gelation by increasing their values. In the case of samples marked 20170831 ABA/ITA-DOPA and 20170822 ABA/ITA-DOPA-Butylamine, the temperature values of the beginning and the ending of sol-gel transition were increased minimally. The curves of described dependence were similar to the lines G´and G´´ of sample 20170329 ABA/ITA.

In the case of sample 20171011 ABA/ITA-DOPA-Butylamine, quite large accruement of both temperatures, initial and final, were reached in comparison to sample 20170329 ABA/ITA. It could be considered as a consequence caused by the presence of aromatic nuclei included by the dopamine´s structure. The aromatic nucleus attached to the end of the polymer chain could behave as a rigid component or its hydroxyl groups could interact with hydrogens to form the hydrogen interactions. The motion of polymer chains was more difficult, the formation of micelles was slowlier and the values of temperatures were increased. The accruement of temperatures accorded with the amount of bonded dopamine. The sample 20170831 ABA/ITA-DOPA and 20170822 ABA/ITA-DOPA-Butylamine comprised less amount of dopamine than the sample 20171011 ABA/ITA-DOPA-Butylamine with the highest amount of bonded dopamine.

It is obvious that the stiffness of gels was decreased significantly when compared to sample 20170329 ABA/ITA.

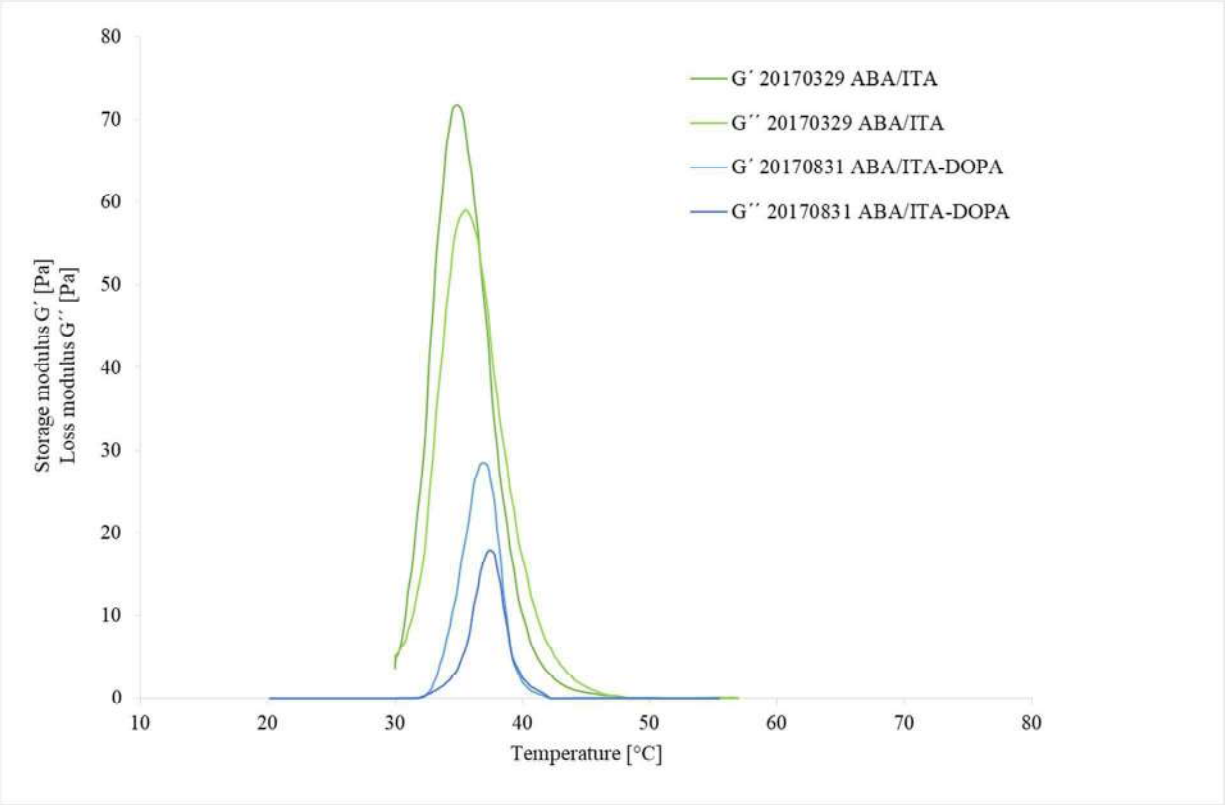

Fig. 43: Rheological properties of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA triblock copolymers.

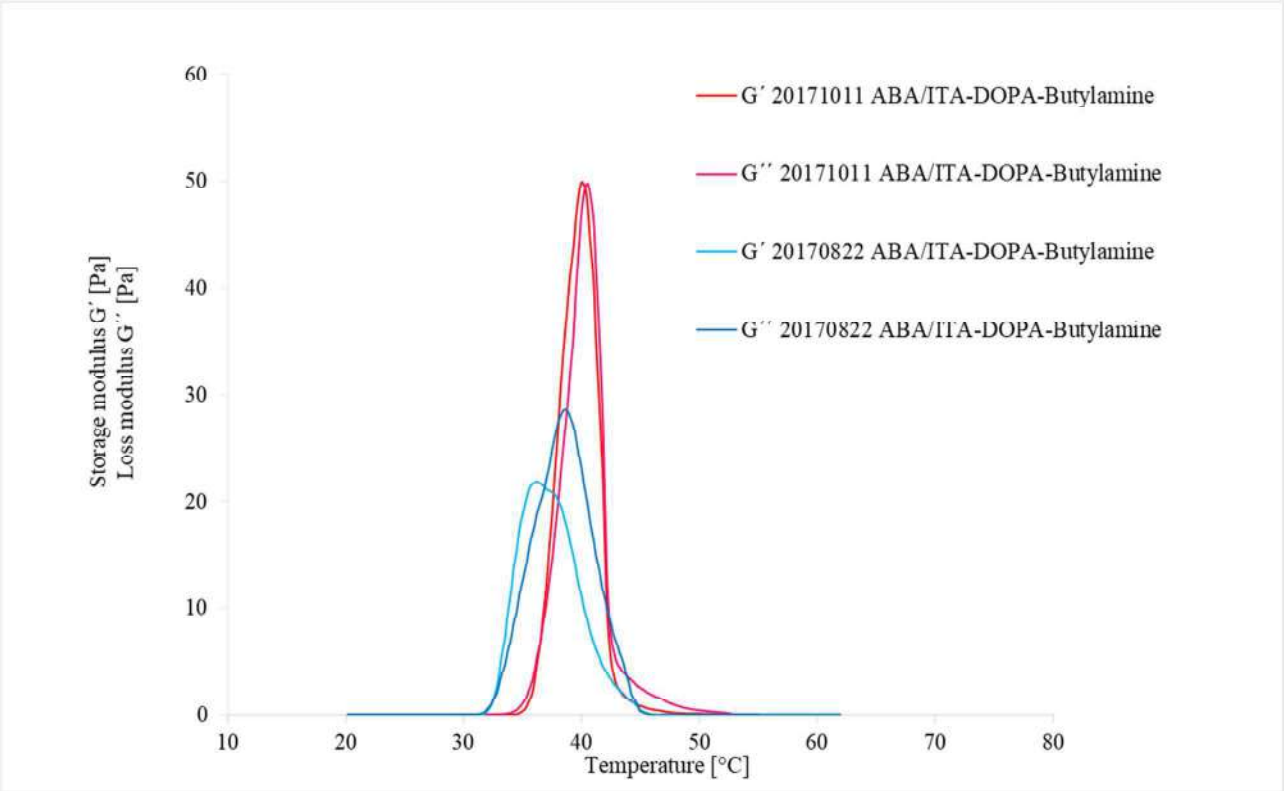

Fig. 44: Rheological properties of two samples of Butylamine-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Butylamine triblock copolymers.

5.2.3 Dopamine and L-lysine Modification in a Bulk

The synthesis of ITA/PLGA-PEG-PLGA/ITA copolymer modified by dopamine was "one-pot" reaction proceeded in a bulk with no solvent used. The first step included the synthesis of triblock copolymer PLGA-PEG-PLGA, the second step involved the functionalization by ITA, the third step was the modification by dopamine and in the last step, after the purification, L-lysine was coupled to dopamine attached to synthesized copolymer (Fig. 45).

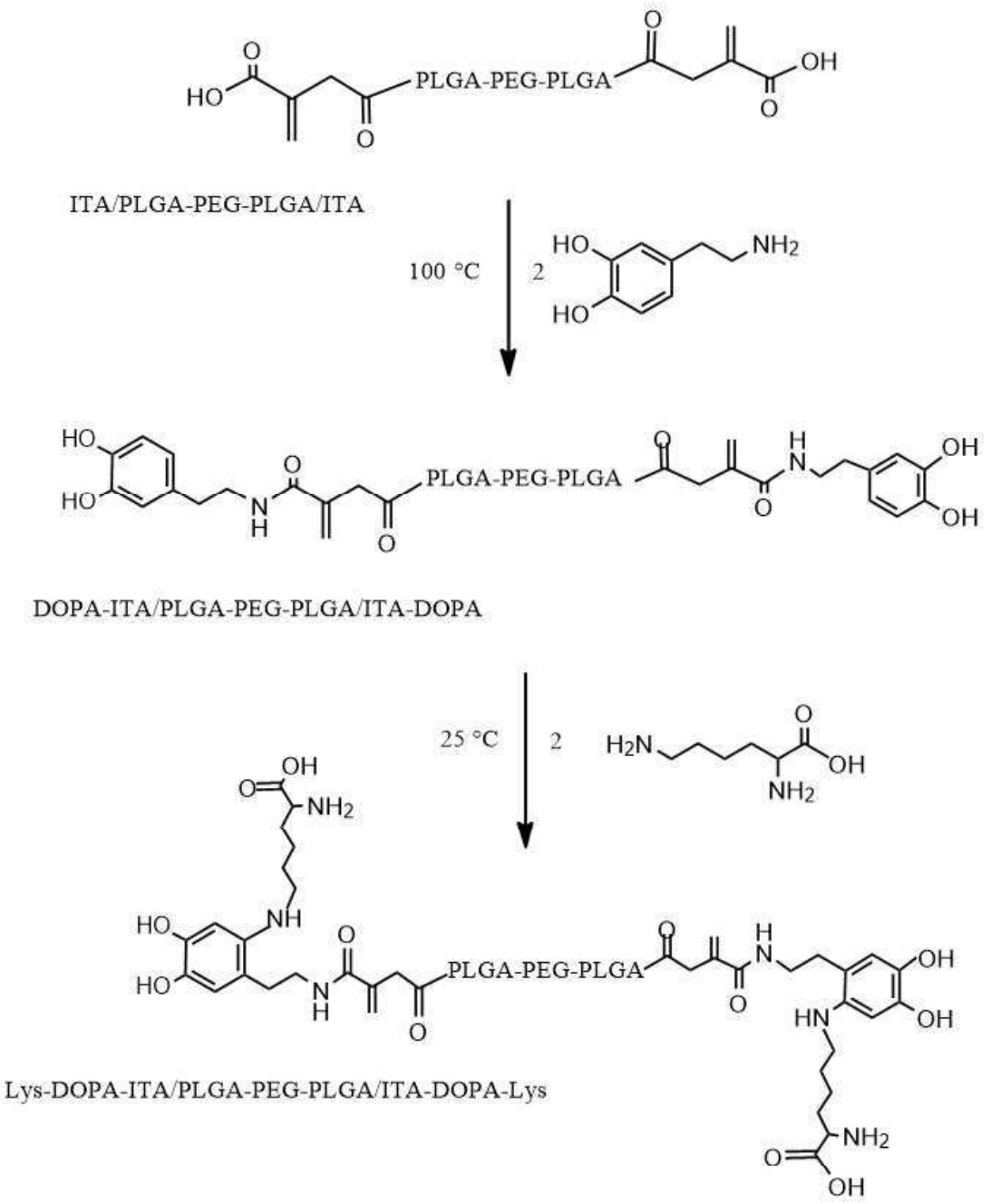

Fig. 45: Synthesis scheme of Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys triblock copolymers.

5.2.3.1 Characterization by Proton Nuclear Magnetic Resonance Spectroscopy

NMR spectrum of copolymers modified by dopamine and L-lysine is showed in the figure 46. Characteristic signals of lactic acid protons (A, E), glycolide acid protons (B), poly(ethylene glycol) protons (D, C) and itaconic acid protons (F, G, H) were observed.

Characteristic signals of lactic acid protons (O-(C<u>H</u>$_3$)C<u>H</u>O) were found in the range between 5.14 - 5.29 ppm (multiplet, 1H) (peak A) and 1.46 - 1.63 ppm (multiplet, 3H) (peak E). Protons of glycolide acid (OC<u>H$_2$</u>O) had characteristic signal in range between 4.61 - 4.92 ppm (multiplet, 2H) (peak B) and signal belonged to protons of poly(ethylene glycol) (OC<u>H$_2$</u>C<u>H$_2$</u>O) was in the range between 3.55 - 3.74 ppm (multiplet, 3H) (peak D) and (-O-CH$_2$C<u>H$_2$</u>-O-) between 4.23 - 4.33 ppm (multiplet, 2H) (peak C).

Characteristic signal of itaconic acid backbone (OC(C<u>H</u>$_2$)CCH$_2$COOH) were found in range between 3.43 - 3.55 ppm (singlet, 1H) (peak H) and protons of itaconic acid double bonds (OC(CH$_2$)CC<u>H</u>$_2$COOH) had signals in range between 5.78 - 5.89 ppm (singlet, 1H) (peak G) and 6.37 - 6.47 ppm (singlet, 1H) (peak F) [2].

Dopamine´s characteristic signals of protons ((OH)$_2$<u>Ph</u>CH$_2$CH$_2$NH$_2$) were expected in the range between 6.69 - 6.80 ppm (multiplet, 3H) (peak I) and ((OH)$_2$PhC<u>H</u>$_2$C<u>H</u>$_2$NH$_2$) protons´ signals ranged from 2.79 to 3.07 ppm (triplets, 4H) (peaks J). Protons of L-lysine (COOH(NH$_2$)C<u>H</u>(CH$_2$)$_3$CH$_2$NH$_2$) could be found at 3.5 ppm (triplet, 1H), (COOH(NH$_2$)CH(C<u>H</u>$_2$)$_3$CH$_2$NH$_2$) protons could be in the range between 1.3 - 1.4 ppm (multiplet, 6H) and (COOH(NH$_2$)CH(CH$_2$)$_3$C<u>H</u>$_2$NH$_2$) protons could be found at 2.6 (triplet, 2H) ppm in according to ^{1}H NMR prediction.

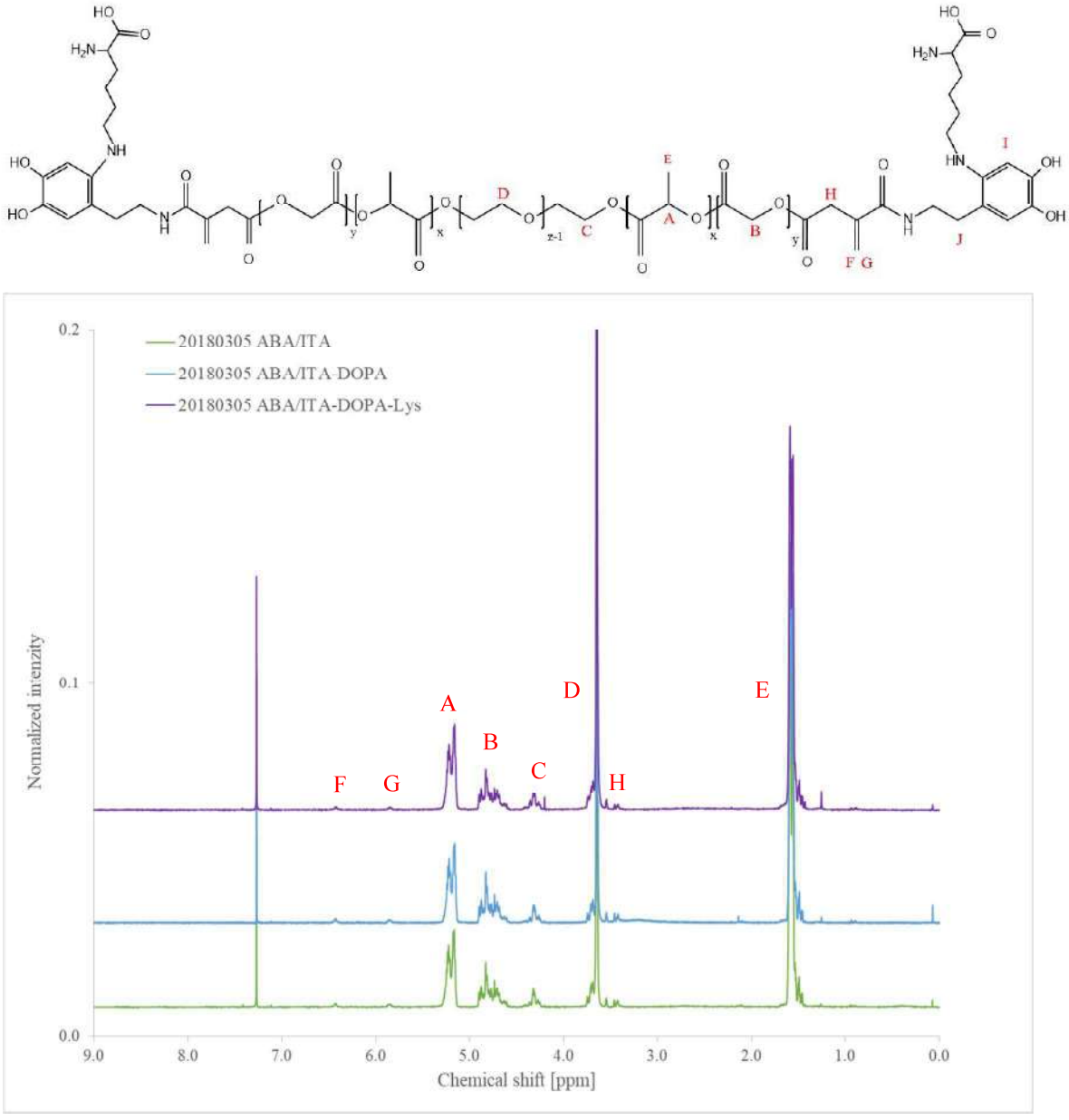

Fig. 46: ^{1}H NMR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA and Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys triblock copolymers.

The presence of dopamine or L-lysine bonded to ITA/PLGA-PEG-PLGA/ITA was not proved, not even in the case of dopamine when compared to NMR spectra of 20170822 and 20171011 shown in

the figure 41. In the spectrum was characteristic signal of solvent $CDCl_3$ with peak at 7.25 ppm. Real molecular weights, PLGA/PEG and LA/GA ratio of synthesized copolymers are compared in the table 7.

Table 7: The comparison of real and theoretical values of observed characteristics.

Sample	ITA	M_n [g mol^{-1}]		PLGA/PEG [wt/wt]		LA/GA [mol/mol]	
	[mol %]	Theor	NMR	Theor	NMR	Theor	NMR
20180226 ABA/ITA	71.1	4500	4587	2.0	2.1	3.0	3.0
20180226 ABA/ITA-DOPA			4588		2.1		3.0
20180305 ABA/ITA			4699		2.1		2.8
20180305 ABA/ITA-DOPA	79.4		4697		2.1		2.7
20180305 ABA/ITA-DOPA-Lys			4699		2.1		2.8

5.2.3.2 Characterization by Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy

Measured infrared spectrum confirmed the outcome of NMR spectroscopy. Besides already identified functional groups belonged to ITA/PLGA-PEG-PLGA/ITA copolymers, ester groups at around 1100 cm^{-1}, carbonyl groups presented by thin peak at 1780 cm^{-1}, characteristic peaks of alifatic alkyl groups observed at 2850 - 2990 cm^{-1} and broad peak of carboxylic groups at around 3450 cm^{-1}, there was observed any other peak characterizing functional group of modified copolymer (Fig. 47).

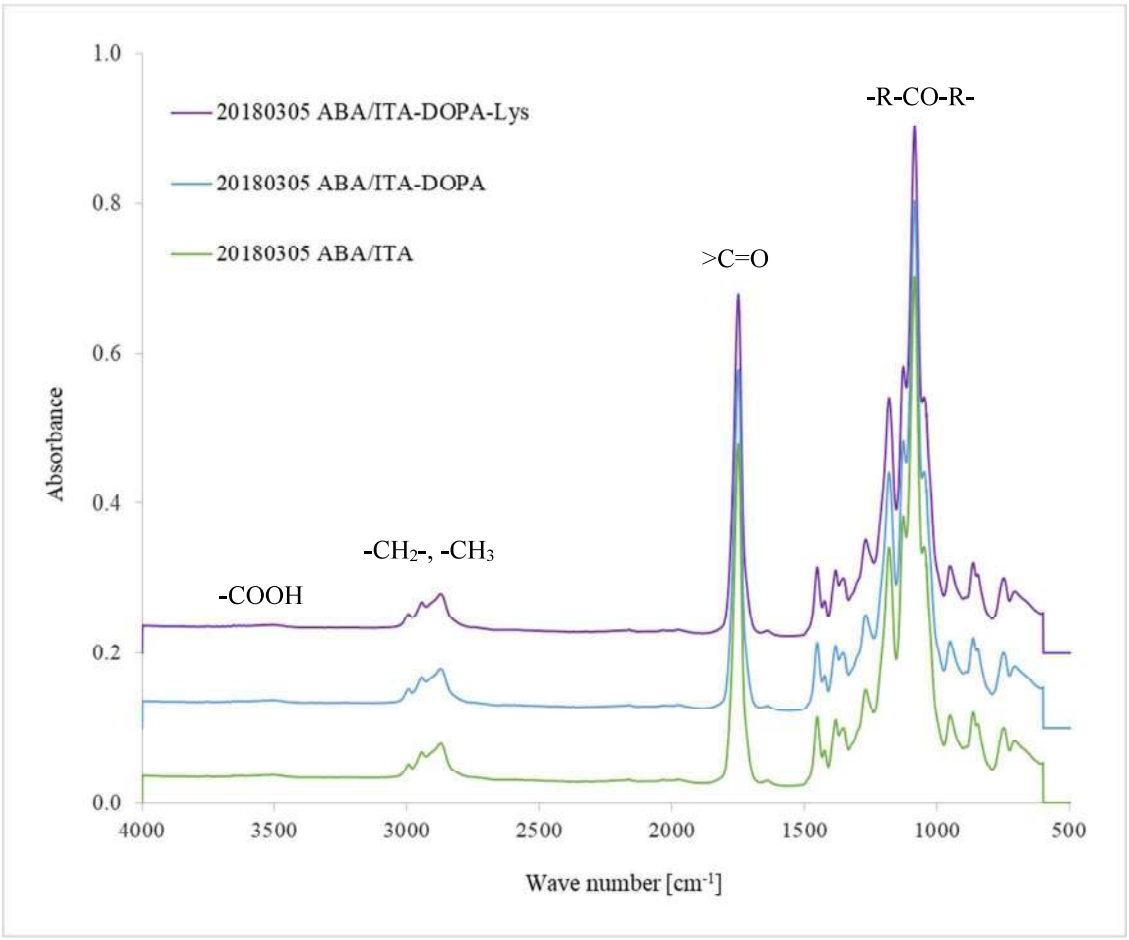

Fig. 47: FTIR spectrum of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA and Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys triblock copolymers.

5.2.3.3 Characterization by Dynamic Rheological Analysis

Dynamic rheological analysis is a method used to study viscoelastic materials. The result of measurement showed the temperature dependent storage and loss modulus. Storage modulus G´ presented the elastic character and the loss modulus G´´ presented the viscous character of material. There were two obtained crosses of storage modulus curve and loss modulus curve in the figure 48.

The first cross of sample 20180305 ABA/ITA-DOPA at 30.3 °C presented the value of gelation temperature, the transition from viscous liquid to solid gel of DOPA modified copolymer ITA/PLGA-PEG-PLGA/ITA (20 % solution in ultrapure water). During the process the temperature and the both modulus increased to the maximum of storage modulus at 115.2 Pa, 35.5 °C and to the maximum of loss modulus at 97.0 Pa, 35.7 °C. The second cross at 36.5 °C showed the end of the gelation process.

The first cross of sample 20180305 ABA/ITA-DOPA-Lys (20 % solution in the ultrapure water) at 68.1 °C was considered to be the critical point of gelation temperature. The maximum of storage modulus was at 49.9 Pa, 40.1 °C and the maximum of loss modulus was at 49.5 Pa, 40.2 °C. The second cross at 84.3 °C was regarded as the end of the gelation process.

The difference in the range of temperatures between the samples 20170329 ABA/ITA and 20180305 ABA/ITA-DOPA was not observed. This finding confirmed the result of NMR analysis. On the other hand, temperature range of the sample 20180305 ABA/ITA-DOPA-Lys was changed markedly.

The stiffness of formed gel was defined by the maximum of storage modulus. It is interesting that the sample 20180305 ABA/ITA-DOPA showed the highest value of storage modulus among all modified copolymers whereas the spectrum measured by NMR analysis proved the absence of bonded dopamine and L-lysine.

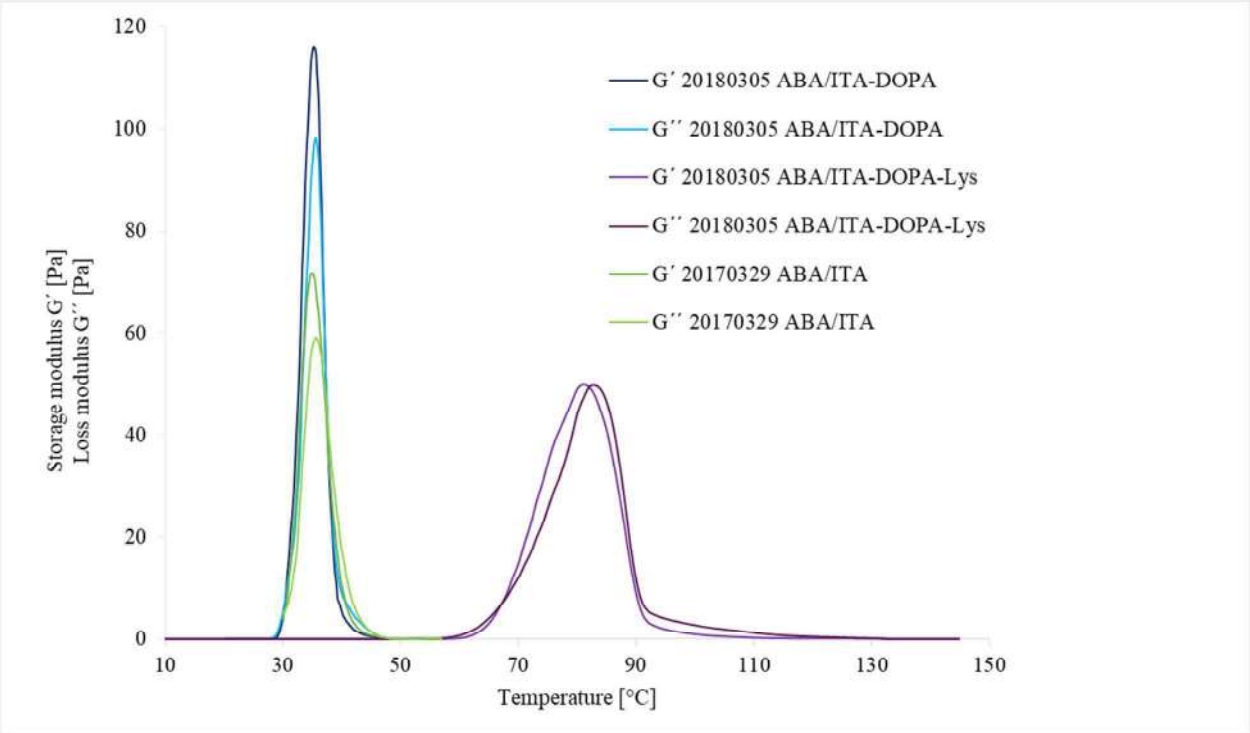

Fig. 48: Rheological properties of ITA/PLGA-PEG-PLGA/ITA and DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA and Lys-DOPA-ITA/PLGA-PEG-PLGA/ITA-DOPA-Lys triblock copolymers.

The summarization of all synthesized samples is presented in the table 8.

Table 8: Summary of synthesized samples.

Number	Sample	ITA [mol %]	DOPA [mol %]	DOPA/ITA [mol %]	ButA [mol %]	ButA/ITA [mol %]	ButA/DOPA [mol %]
20170306	ABA/ITA	51.5	-	-	-	-	-
20170306	ABA/ITA-DOPA	49.7	-	-	-	-	-
20170720	ABA/ITA-DOPA	-	-	-	-	-	-
20170329	ABA/ITA	76.2	-	-	-	-	-
20170831	ABA/ITA-DOPA	76.2	7.7	10.1	-	-	-
20170822	ABA/ITA-DOPA-ButA		8.5	11.2	4.5	5.4	48.0
20171010	ABA/ITA	57.4	-	-	-	-	-
20171011	ABA/ITA-DOPA-ButA		18.6	32.4	7.8	13.5	41.8
20180226	ABA/ITA	71.1	-	-	-	-	-
20180226	ABA/ITA-DOPA		-	-	-	-	-
20180305	ABA/ITA	79.4	-	-	-	-	-
20180305	ABA/ITA-DOPA		-	-	-	-	-
20180305	ABA/ITA-DOPA-Lys		-	-	-	-	-

6 CONCLUSION

The main aim of the diploma thesis was modification of ITA functionalized biodegradable thermoresponsive copolymer PLGA-PEG-PLGA. The functionalization by itaconic acid brought new options to improve mentioned copolymer. Itaconic acid functionalized both ends of the copolymer by reactive double bonds and carboxylic groups. The double bonds enabled to form chemical cross-links and the end-capped carboxylic groups offered the modification by biologically active compounds.

Firstly, functionalized copolymer ITA/PLGA-PEG-PLGA/ITA was synthesized in order to obtain reactive double bonds and carboxylic groups suitable for consequent modification. The copolymerization consisted of two steps proceeded via catalytic ring-opening mechanism in a bulk. The first step involved the synthesis of triblock copolymer PLGA-PEG-PLGA and the second step involved its functionalization by ITA. Altogether, 5 samples of ABA/ITA was synthesized and characterized by ^{1}H NMR, FTIR and DRA. Molecular weight calculated from NMR spectra was consistent with theoretical molecular weight of copolymers. The amount of bonded ITA ranged between 51.5 - 79.4 mol %. Higher amount of bonded ITA was reached during more intensive stirring of viscous reactive mixture.

The second part of diploma thesis was focused on the attachment of dopamine, bioactive, adhesive and stabilizing agent. The attachment was carried out in aqueous solution, organic solution and in a bulk. The synthesis differed in the usage of activating systems. The third step of modifying synthesis was linking of the butylamine and L-lysine as a bioactive compounds with relatively simple structure that replaces the complex structure of proteins.

At the beginning, the synthesis of dopamine modified triblock copolymer ITA/PLGA-PEG-PLGA/ITA was proceeded in aqueous solution with and without activating system EDC/NHS. Two experiments were unsuccessfull because dopamine tended to form polydopamine. It was obvious by changing the colour of samples from white to black and ^{1}H NMR, FTIR or DRA analyses contradicted the appearance of dopamine or peptide bond in the samples.

Subsequently, the modification of triblock copolymer ITA/PLGA-PEG-PLGA/ITA by dopamine continued by the reaction in the organic solution with solvent DMF in the presence of activating system DCC/DMAP. The successfull dopamine modification was extended by the third step including the attachement of butylamine. Overall, 3 samples of ABA/ITA-DOPA was synthesized and characterized by ^{1}H NMR, FTIR and DRA. The amount of bonded dopamine in the sample labeled 20170831 was 7.7 mol % and in the proportion to bonded ITA it occupied 10.1 mol %. The amount of bonded dopamine in the sample marked 20170822 was 8.5 mol % and in the proportion to bonded ITA it was 11.2 mol %. The sample labeled 20171011 had the amount of bonded dopamine at value 18.6 mol % and in the proportion to bonded ITA it occupied 32.4 mol %. The synthesis of last sample was optimized by prolonging the activation time of end-capped carboxylic functional groups. Samples 20170822 and 20171011 were also modified by butylamine. The amount of bonded butylamine in the sample labeled 20170822 was 4.5 mol % and in the proportion to bonded ITA it occupied 5.4 mol %.

The amount of bonded butylamine in the sample marked 20171011 was 7.8 mol % and in the proportion to bonded ITA it was 13.5 mol %. The proportional amount of butylamine to bonded dopamine occupied 48.0 mol % for sample 20170822 and 41.8 mol % for sample 20171011. FTIR spectra of all samples proved the presence of peptide bonds. DRA measurements founded the accruement of both temperatures, the temperature of forming gel and the temperature of ending the gelation process. The largest accruement was noticed at the sample labeled 20171011 with the highest amount of bonded dopamine. It could be the consequence caused by the presence of aromatic nuclei involved by the dopamine´s structure. The aromatic nucleus attached to the end of the polymer chain could behave as a rigid component or its hydroxyl groups could interact with hydrogen to form the hydrogen interactions. The motion of polymer chains was more difficult, the formation of micelles was slowlier and the values of temperature were increased.

Following experiments was proceeded in a bulk to try the synthesis with no orgnic solvents and compounds used. The first step included the synthesis of triblock copolymer PLGA-PEG-PLGA, the second step involved the functionalization by ITA, the third step was the modification by dopamine and in the last step, after the purification, L-lysine was coupled to bonded dopamine in aqueous polymer solution. Altogether, 3 samples were prepared. The results obtained from ^{1}H NMR, FTIR or DRA analyses did not prove the presence of bonded dopamine or L-lysine in synthesized samples.

At the close of work, noticeable progress is summarized. The synthesis of functionalized triblock copolymer ITA/PLGA-PEG-PLGA/ITA was optimalized and it was managed to reach higher amount of bonded ITA up to 79.4 mol %. High amounts of bonded ITA were reached by more intensive stirring of viscous reactive mixture.

Experiments in aqueous solution showed the impropriety of manipulation with dopamine in the presence of water because of its tendency to form polydopamine.

The effectual method of synthesis DOPA modified triblock copolymer ITA/PLGA-PEG-PLGA/ITA was found. The synthesis in the environment of organic solvent DMF and in the presence of activating system DCC/DMAP was resultful. The highest efficiency was reached in the synthesis of sample 20171011 ABA/ITA-DOPA-Butylamine because of longer activation time.

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LIST OF ABBREVIATIONS AND SYMBOLS

CGC	Critical gel concentration
CGT	Critical gel temperature
ABA	PLGA-PEG-PLGA
BAB	PEG-PLGA-PEG
LA	Lactic acid
GA	Glycolic acid
PLGA	Poly(D,L-lactic-co-glycolic acid)
PLA	Poly(lactic acid)
PGA	Poly(glycolic acid)
T_g	Glass transition temperature
FDA	Food and Drug Administration
PEG	Poly(ethylene glycol)
SA	Succinic anhydride
SAP	Poly(ethylene glycol succinate)
RIF	Rifampicin
FA	Folic acid
PCL	Polycaprolactone
CAP	Capecitabine
VCR	Vincristine sulfate
ITA	Itaconic anhydride
DOPA	Dopamine
ADHD	Attention deficit hyperactivity disorder
Lys	L-lysine
ButA	Butylamine
Acetyl-CoA	Acetyl-CoenzymA
RGD	Arginine-glycine-aspartic acid peptide sequences
FGF	Fibroblast growth factors
bFGF/FGF 2	Basic fibroblast growth factor
Sn-octoate	Sn(II)2-ethylhexanoate
EDC	N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride
NHS	N-Hydroxysuccinimide
DMF	N,N-Dimethylformamide
DCC	Dicyclohexylcarbodiimide
DMAP	4-(Dimethylamino)pyridine
DEE	Diethyl ether
^{1}H-NMR	Proton Nuclear Magnetic Rezonance Spectroscopy
ATR-FTIR	Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy
DRA	Dynamic Rheological Analysis
Theor	Theoretical
M_n	Molecular weight

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